The Analysis of Caffeine in oral fluid using Surface-Enhanced Raman Spectroscopy and High-Performance Liquid Chromatography

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Supervised by Dr. Mark Baron.

i. Certificate of originality

This is to certify that I am responsible for the work submitted in this thesis, that the original work is my own, except as specified in the acknowledgements and in references, and that neither the thesis nor the original work contained therein has been previously submitted to any institution for a degree.

Signature:

Name:

Date:

ii. Acknowledgments

I would like to take this opportunity to show a huge amount of gratitude to the people who have helped me throughout this research project. Firstly to my supervisor Dr Mark Baron for his guidance and trust in my competence of completing this project to his standard and finally I would like to give my husband Mark Andrew a massive thankyou for having to put up with me through this stressful but enjoyable task especially the early morning lab sessions.

iii. Abstract

Surface-Enhanced Raman spectroscopy (SERS) is a technique that is preferred over conventional Raman spectroscopy. The main advantage of SERS is that the intensity of the Raman signal is enhanced up to 10^4 to 10^6 fold thus allowing low concentrations of the analyte to be detected. The use of oral fluid for detecting drugs of abuse is increasing in popularity as the methods of collecting saliva are not as invasive as other biological fluids (blood or urine). As SERS can detect low limits of substances, the use of this technique coupled with the analysis of oral fluid for the detection of drugs is becoming popular. This technique could have applications in roadside testing, drug testing and medical care. The constant appearance of caffeine present in new psychoactive substances is becoming increasingly common. There are a lot of the drugs that are being sold on the market that only contain caffeine rather than the stated drug. A market is opening up for the detection of drugs in saliva using a rapid portable testing kit. This work focuses on the analysis of caffeine as it is a common adulterant that it found in illegal and legal drugs of abuse. A reproducible silver colloid with strong surface-enhancement and good shelf life was used to optimise a method capable of analysing caffeine. The colloid was produced by the reduction of silver nitrate with hydroxylamine phosphate. An optimised SERS method was developed that was capable of detecting caffeine in methanolic solutions, however when analysing caffeine in saliva there were problems that meant a spectrum could not be obtained. A HPLC method was developed for the quantification of caffeine. The HPLC method was successfully able to detect and quantify caffeine in saliva using 8 independent participants, the participants were asked to go 24 hours without any form of caffeine, and they were then required to give a saliva sample before and after the consumption of caffeine. The caffeine concentrations ranged between 1.51-1.94 μg/mL. The HPLC results show that caffeine was present at levels that should have been detected by the SERS method. Further investigation is needed to determine why SERS couldn't detect caffeine in saliva; this could be due to other substances present in saliva.

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1. Introduction

There are a wide variety of different analytical techniques that are being used to analyse drugs of abuse and legal highs. The most common technique used is gas chromatography-mass spectrometry (GC-MS) as it is sensitive and selective, other techniques that have been developed are thin-layer chromatography (TLC), high performance liquid chromatography (HPLC) and immunoassays. HPLC is normally a secondary choice however HPLC can be applied to water soluble compounds which are increasing rapidly within the toxicological field (Nakashima, 2005).

One method that has been looked at to determine specific drugs within saliva samples is surface-enhanced Raman spectroscopy (SERS) (Shende et al., 2005). The main advantage of using SERS is that unlike immunoassays it is not subject to interferences that can lead to false positives. A further advantage of SERS is that when using sophisticated instruments like GC-MS to identify and quantify illicit drugs the analysis is usually time-consuming and labour-intensive (Wood et al., 2003). SERS however, only takes a few minutes. The use of saliva is also beneficial as again it is non-invasive, samples are easy to collect, drugs are represented in concentrations similar to blood plasma (μg/mL) and as saliva is 99.5% water the number of steps required to remove any chemical interferences is significantly fewer (Farquharson et al., 2005).

High performance liquid chromatography (HPLC) is becoming an increasingly popular chromatographic method used to analyse different drugs of abuse and adulterants especially within saliva. The use of oral fluid for the analysis of drugs of abuse is an attractive alternative to plasma and urine which are currently the most commonly investigated. Collection of saliva is non-invasive and requires no specialist equipment and can be supervised, removing sample contamination which is of great importance for forensic toxicologists.

The aim of this research is to be able to identify caffeine within oral fluid by SERS using a silver colloid and quantify using a HPLC method.

2. Literature Review

2.1 Caffeine

Caffeine is a xanthine alkaloid that is used as a stimulant drug. It has a melting point of 235-238°C, is moderately soluble in water at room temperature (2g/100 mL) however, very soluble in boiling water (66 g / 100 mL). It is weakly basic with a pK_a of 0.6. Caffeine is classified as an achiral molecule as it does not contain any stereogenic centres (Chen, Gu, Shen, Dong, & Kang, 2010). The core of a caffeine molecule contains two fused rings; pyridine (6-membered) ring and imidazole (5 membered) ring (Chen et al., 2010) (figure 1). The pyridine ring alone contains two amide groups and two tertiary amine function groups. The imidazole ring contains one amide group and one tertiary amine, along with an alkene functional group (Chen et al., 2010).

It can be found in different foods and plants. It is often consumed in the form of tea, coffee, energy drinks etc. In the human body it acts as a central nervous system (CNS) stimulant by restoring alertness, preventing drowsiness and helps increase cognitive function (Tabassum et al., 2012). It is the world's most widely consumed psychoactive drug that is legal. Within 30-40 minutes of consumption caffeine reaches its highest concentration in the blood and brain and caffeine has a half-life of 4-6 hours (Tabassum et al., 2012).

Caffeine is commonly found as an adulterant in street drugs such as amphetamine, cocaine, heroin, ecstasy and sympathomimetic recreational drugs (NPS) (Davies et al., 2012; Vanattou-Saïfoudine et al., 2012). However as there is often limited information regarding the contents of these products they could contain other compounds such as caffeine. It is plausible that caffeine could serve to promote these reactions when taken in combination with illicit psychostimulants. The harmful effects caffeine has when consuming other psychostimulants is becoming problematic as many controlled and uncontrolled drugs contain some caffeine. Table 1 shows different papers that have been published where caffeine was found mixed with other drugs and NPS or caffeine was the main active ingredient.

Table 1: List of different drugs and NPS where caffeine was found

References	Ingredient stated	Main Active Ingredient	Caffeine present
Baron et al (2011)	MDAI	Caffeine	Yes
	5-IAI	5-IAI	Yes
	NRG-3	6-APB	Yes
Davies et al (2012)	Legal High	Caffeine	Yes
	Legal High	Caffeine	Yes
	Legal High	Caffeine	Yes
Vanattou-saïfoudine et al (2012)	MDMA	MDMA	Yes
	Amphetamine	d-amphetamine	Yes
O'Bryne et al (2013)	Methedrone	Methedrone	Yes
	Fluoromethcathinone	Fluoromethcathinone	Yes
Brant et al (2010)	NRG-1	Caffeine, mephedrone traces	Yes
	DMC	Caffeine, lidocaine	Yes
	NRG-1	Caffeine	Yes
	NRG-2	Benzocaine	Yes
	NRG-1	Caffeine	Yes

Table 1 demonstrates that a lot of drugs that are being sold may only contain caffeine particularly in the sale of NPS. If people are taking a drug that they believe to have a psychoactive substance in but only contains caffeine and do not feel any effect from the drug then they could possibly take more which could result in a caffeine overdose.

Over 300 people consume caffeine on a daily basis (Vanattou-Saïfoudine et al., 2012). The daily intake normally ranges from 50 mg - 150 mg (Vanattou-Saïfoudine et al., 2012). Caffeine overdoses have been associated with oral consumption of between 3 and 20 g (Vanattou-Saïfoudine et al., 2012). It has been reported that caffeine has the potential to influence the toxicity of other stimulants. Derlet et al (1992) administered caffeine and amphetamine or cocaine to rats and it was found that it resulted in seizures and mortality when compared to the administration of amphetamine or cocaine alone. It has also been

demonstrated more recently when caffeine and MDMA were administered to rats that it caused seizures, tachycardia and hyperthermia (McNamara et al., 2007).

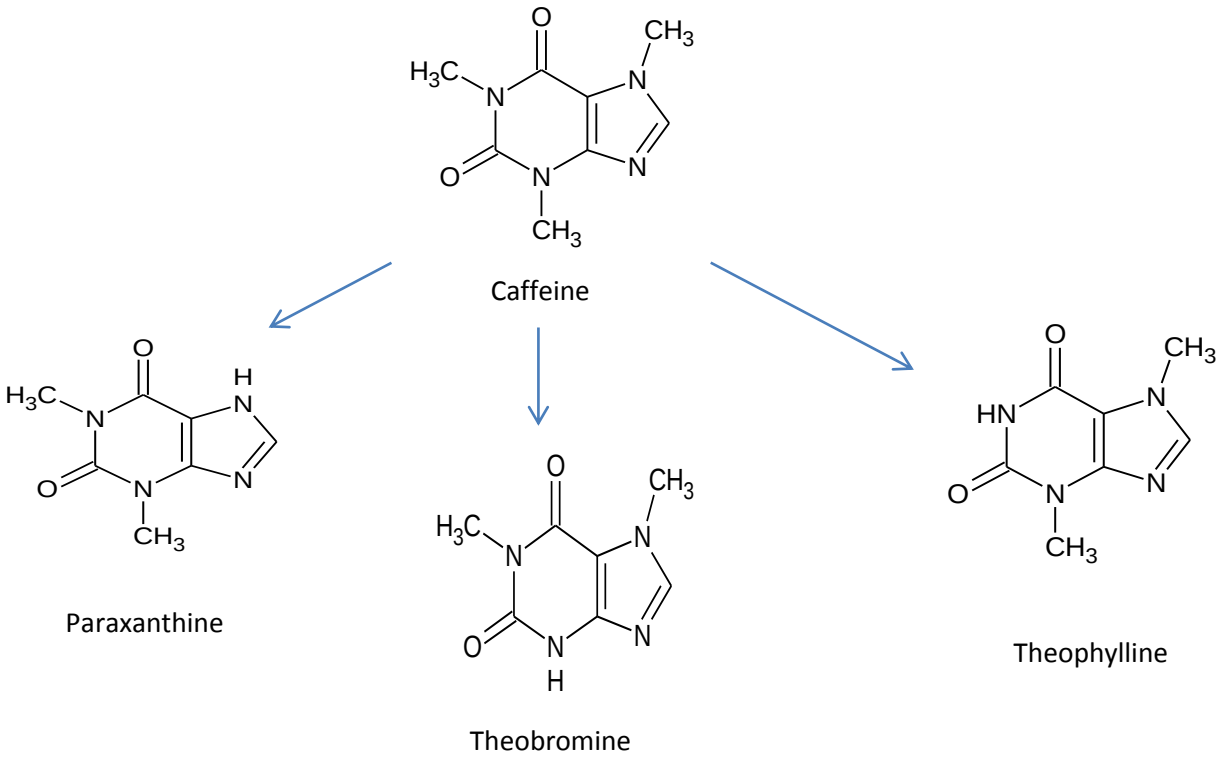
There have been a number of reports of the toxicity of caffeine. In 2014, Smith, reported that an 18 year old girl on a night out had drunk Jagerbombs (mixture of Jagermeister spirit and caffeine-filled energy drinks). Eight hours after she had consumed these drinks she collapsed in her bathroom at home. Her family had to perform CPR three times in order to keep her alive. Once she was admitted to hospital she was put in a coma for 52 hours to protect her brain and heart. The medics informed the family that when the alcohol she had drunk had worn off the caffeine in her system took control of her heart beat. To ensure her heart beat remained normal a defibrillator was implanted under her skin to shock her heart back to a normal rhythm. Doctors have informed people that children and young adults are at a potential risk from high amounts of energy drinks as their smaller bodies are not used to the effects and it can cause heart palpitations. It has also been reported that a 40 year old man died last year from a caffeine overdose after eating a pack of 12 energy mints (Smith, 2014).

Inside the body caffeine counteracts adenosine (Fredholm, 2011a). Adenosine acts as an inhibitory neurotransmitter that suppresses activity in the CNS, as caffeine readily crosses the blood-brain barrier it blocks the adenosine receptors as it is structurally similar and acts as a competitive inhibitor preventing drowsiness (Fredholm, 2011a). Caffeine also interacts with dopamine which could be the cause of caffeine's addictive nature (Alpert, 2012).

There are three metabolites of caffeine; paraxanthine, theobromine and theophylline. Caffeine is metabolised primarily by demethylation in phase 1 liver detoxification by the CYP450 system, specifically 1A2 (Alpert, 2012; Benowitz et al., 1995) which can be seen in Figure 1. Over 80% of caffeine administered metabolises to paraxanthine, 16% is converted to theobromine and theophylline (Xu et al., 2010). Paraxanthine increases the lipolysis process, releasing glycerol and fatty acids into the body as a fuel source for muscles (Alpert, 2012). Theobromine is predominantly found in chocolate and can also be found in small quantities in tea (Fredholm, 2011b). Theobromine increases the amount of oxygen and nutrient flow to the brain and muscles by dilating the blood vessels (Alpert, 2012).

Theophylline acts as a smooth muscle relaxant that increases the heart rate and force of contraction and is widely employed in the treatment of bronchial asthma (Alpert, 2012). These metabolites are then broken down further and excreted in urine (Alpert, 2012).

Figure 1: metabolism of caffeine in the liver.



Caffeine is commonly used in psychoactive substances as either a replacement for the active drug or as a cutting agent (table 1). Being able to detect caffeine in saliva may also result to being able to detect different psychoactive substances as well.

2.2 New psychoactive substances

New psychoactive substances (NPS) are commonly known as 'legal highs' and are increasingly being used throughout European countries (Davies et al., 2012). The Government has openly stated that just because these substances are called 'legal highs' it does not make them safe to use as they have not been subjected to the stringent testing procedure which are required before they are granted a licence (ACMD, 2011). NPS normally fall broadly into four categories (ACMD, 2011):

- Products with names which give no indication of what they contain
- Named and specific substances which are designed to be similar chemically/pharmacologically to known controlled drugs
- Substances related to medicines
- Herbal and fungal materials

They are sold either on the internet or in head shops. Generally, these substances contain stimulant drugs that mimic the effects of drugs that have been controlled such as amphetamine and ecstasy. Synthetic substances have increased in popularity which are causing problems for authorities as they are unable to act quickly enough to monitor and legislate the vast array of substances (Ayres et al., 2012) Due to an increase in these substances there has been a corresponding increase in published papers reporting on their adverse effects (Gibbons, 2012). Their chemical composition, toxicity and their long-term effects pose a challenge to public health agencies as little is known about them. Many of these substances have already been categorised under the Misuse of Drugs Act for example mephedrone and a group of spice compounds that have a specific chemical structure. However, despite these controls it is evident that these banned substances are still being sold under different names (Ayres et al., 2012). Scientists are unsure of what products contain newly synthesised drugs and how many contain controlled substances. In order to protect people from the harmfulness of these NPS's, a temporary class order was introduced in 2011 by the Advisory Council on the Misuse of Drugs Act, 1971 (ACMD). This enables the ACMD to put a temporary ban on new 'legal highs' for 12 months while health issues regarding these substances are considered. An example of where this has already been put in place is with methoxetamine (MXE) (Ayres et al., 2012). Methoxetamine's chemical structure resembles that of ketamine which is a Class C drug. It has been reported that the effects of methoxetamine are similar to ketamine; severe dissociation, paranoid thoughts, hallucinations and cardiovascular symptoms. However it has also been reported that the effects of MXE are of a longer duration. The ACMD recognises that the effects of MXE are commensurate with Class B substances and it should be scheduled under Schedule 1 of the Misuse of Drugs Regulations (2001) (ACMD, 2012). Since then a new act has been brought in, the Psychoactive Substance Act (2016). This Act has been brought in to restrict the production, sale or supply of psychoactive substances and is not intended to criminalise

possession. However it does criminalise supply, so head shops and internet sites are unable to supply them anymore. There are three exceptions which are caffeine, alcohol and nicotine (Home Office, 2016).

Many of these NPS are being substituted with other adulterants for example caffeine (Davies et al., 2012). Davies et al (2012) looked at the risk of caffeine toxicity associated with the use of NPS. They looked at six different novel psychoactive substances purchased from internet suppliers. They concluded that all six substances contained caffeine; no other active compounds were identified in the screening procedure. They found that there was a wide variation of caffeine. Four of the substances had high proportions of caffeine at 87, 94, 94 and 96% equating to more than 870 mg of caffeine in 1 gram of product. This study shows that people who do buy NPS are at risk of caffeine toxicity related to the high caffeine content. They suggested that a potential reason caffeine was used instead of the intended NPS was to mimic the stimulant effects of the said NPS. It could also have been possible that the suppliers did this because caffeine is much cheaper.

2.3 Drug screening analysis

Delivering accurate and precise chemical analysis on different legal and illegal drugs of abuse is becoming difficult as the work load in forensic labs continues to increase (Stoll et al., 2006). This is encouraging the development of analytical techniques that can provide high quality quantitative and qualitative analyses. No single technique has an overwhelming predominance for screening analysis; the majority of cases require a number of different techniques.

Gas chromatography using flame ionisation detection (GC-FID) was used for screening purposes (Foerster et al., 1978) however gas chromatography mass spectrometry (GC-MS) has replaced the use of GC-FID and is currently considered as the 'golden-standard' technique for drugs of abuse analysis (Øiestad et al., 2007; Stoll et al., 2006). GC-MS is a well-established technique that provides high resolution separation, high selectivity and sensitivity (Pragst et al., 2004). However, there are several limitations for using this analysis as a screening technique. The mass spectrometer as a detector is expensive and can be

unavailable to labs with limited resources. Another limitation with the GC-MS is that not all analytes can be distinguished. A study done by Elie et al (2012) found that when analysing a number of novel psychoactive substances, 5- and 6-APB coelute. A way to combat this problem is by using derivatisation, which increases the volatility of analytes and changes their mass spectra, which in turn affects the retention time. Derivatisation can be costly, time consuming, and susceptible to errors that can affect the quality of the results (Leinonen et al., 2002).

Due to limitations cited above liquid chromatography is looked at to resolve some of them. High performance liquid chromatography (HPLC) has been used as a complementary method to the GC as it separates a wider range of compounds without the need for pre-treatment for example derivatisation (Concheiro et al., 2005). HPLC requires a detector normally a diode-array detector (DAD) which records the absorbance of compounds and produces a UV spectrum (Elliott & Hale, 1998). HPLC-DAD is a very important tool in the identification of substance. With HPLC some classes of drugs can exhibit similar UV spectra, therefore it is desirable to have a second parameter namely the retention time (Elliott & Hale, 1998).

2.4 Chromatographic Techniques

2.4.1 High Performance Liquid Chromatography (HPLC)

HPLC has commonly been a secondary choice in comparison to GC-MS. However, HPLC can be applied to water-soluble compounds making it essential for forensic and toxicological fields. The development of these HPLC methods for analysing drug-related substances has rapidly increased within the last 10 years (Nakashima, 2005). HPLC works by passing a liquid sample through a column containing a solid adsorbent material. Different analytes interact differently with the column, thus delaying the flow of the analytes. With weak interaction, the analytes elute off the column in a short time, and with strong interaction, the elution time is longer.

HPLC uses many different detectors, such as ultraviolet (UV), electrochemical (EC), fluorescence (FL), diode array (DAD) or mass spectral (MS) (Nakashima, 2005; Saxon et al., 1988). HPLC is an alternative technique to GC that is very useful for compounds that are

polar and thermolabile. This method is rapidly growing in the detection of abused drugs due to its simple sample preparation (Tsai et al., 2013).

Table 2: Previous HPLC methods used for the detection of drugs of abuse in biological fluids

References	Analytes Validated	Column	Mobile Phase	Flow rate (mL/min)	Detection wavelength	Run time (min)
Alvi et al (2011)	Caffeine	C_{18}	Potassium Phosphate – acetonitrile (83:17)	1.0	274 nm	10
Tsai et al (2013)	Drugs of abuse and metabolites	EC-C_{18}	Acetic acid – Methanol	0.4		14
Perera et al (2010)	Caffeine Paraxanthine	C_{18} Ultrasphere ODS	Acetonitrile – acetic acid – water (100:1:899)	1.5	280 nm	15
Mumin et al (2006)	Caffeine		Potassium dihydrogen phosphate – acetonitrile - methanol (40:8:2)	0.5	254 nm	

Alvi et al (2011) devised a method for the determination of caffeine levels in human plasma using the HPLC. They found that caffeine had a retention time of 4.7 minutes and the run time took 10 minutes. Two hundred microliters of the internal standard (antipyrine) was added to 500 µl of the plasma samples. The samples then underwent vortexing and centrifugation at 12000 rpm. The supernatant was collected and injected into the chromatographic system. They were able to determine the limit of quantitation using caffeine free plasma. It was concluded that the limit of quantitation was 0.05 µg/mL and the lowest detection limit was 0.02 µg/mL. Plasma samples from volunteers were analysed using this HPLC method, the mean caffeine level for these volunteers was 0.28 µg/mL after abstaining from beverages and food containing caffeine for 16 hours. Their plasma was then analysed 3 hours after the ingestion of 300 mg of caffeine.

Tsai et al (2013) developed a simple and effective HPLC method that was coupled with quadrupole time-of-flight mass spectrometry to screen and confirm abused drugs. This method was used to analyse 10 urine samples of patients that were undergoing methadone therapy. They were also able to analyse 62 abused drugs and their metabolites. The results obtained showed good selectivity, precision and detection sensitivity.

Another study used HPLC for the analysis of caffeine in saliva (Perera et al., 2010). They looked at the analysis and stability of caffeine and paraxanthine in saliva and plasma. For the development of their study they used concentrations of 1.0 mg/mL for paraxanthine and 2.5 mg/mL for caffeine as these represent the C_{max} values observed in a single oral dose of 100 mg of caffeine. Perera et al (2010) also investigated the stability of these two substances in oral fluid. The two different conditions that were investigated were refrigerated samples at 4^{o}C and room temperature ranging from 9-30^{o}C. They concluded that paraxanthine and caffeine were stable at both temperatures over a 14 day period and the concentration did not vary. Two healthy, non-smokers received a caffeine dose of 100 mg after a 24 hour abstinence and their saliva and blood were collected at different intervals over a 24 hour period. They found that caffeine eluted at 10.5 min, paraxanthine at 5.5 min and the internal standard (benzotriazole) at 14.2 min. The concentration of both caffeine and paraxanthine fell within the validated range over the 24 hour period. The C_{max} value for caffeine in saliva was 1.2 and 1.9 µg/mL for participant 1 and 2 respectively and the half-life was 5.9 and 4.9 respectively.

2.5 Raman Spectroscopy

Raman scattering occurs by a shift in photon frequency which is a result of excitation or deactivation of molecular vibrations i.e. the photon may either gain energy causing a shift up or lose energy causing a shift down (Das & Agrawal, 2011). Raman spectroscopy is becoming increasingly popular as it is possible to acquire spectra quickly, easily and is affordable. In many cases Raman spectroscopy has been coupled with different analytical techniques such as, HPLC, microchromatography, atomic force microscopy and many more (Das & Agrawal, 2011).

Two main features of Raman spectroscopy which make it a useful method for screening tablets/powders are, the ability to record spectra with no sample preparation and the short analysis time (Bell et al., 2000). The spectra not only can identify the active drugs they can also identify excipients, the relative concentration of the drug to excipient and the degree of hydration. This gives Raman an edge in comparison to commercial GC-MS where this technique can identify and quantify the active compound but doesn't give any other information on tablet composition (Bell et al., 2000). Raman spectra can be acquired rapidly suggesting that it would be able to analyse hundreds of samples in just a few hours. In contrast, only a small number of individual tablets are analysed with GC-MS due to time required for each measurement (Bell et al., 2000).

Though Raman spectroscopy has many advantages over other analytical techniques, it also comes with its own limitations. One limitation of Raman spectroscopy is that signals obtained by normal Raman are usually very weak (Das & Agrawal, 2011). This would be problematic if low limits of detection are being assessed. Another limitation is that there is a possibility that if mixtures are being analysed then their spectra could overlap.

2.5.1 Surface Enhanced Raman Spectroscopy (SERS)

SERS enhances the Raman scattering signal by adsorbing molecules onto a roughened metal surface. This enhancement method was first studied in 1977 by Jeanmaire et al and Albrecht et al. Two mechanisms were proposed for the observed enhancement:

- Electromagnetic enhancement, associated with the roughened metal surface (Jeanmaire et al., 1977)
- Chemical enhancement, due to the electronic coupling of molecules adsorbed on the roughened metal surfaces (Albrecht et al., 1977)

Electromagnetic enhancement works on the basis that there is excitation on the metal surface by the surface plasmons resulting in resonance (Chen et al., 2010). The chemical enhancement works due to the analyte forming bonds with the metal surface creating new electronic states due to the different formation of the bonds (Chen et al., 2010).

The signal obtained by normal Raman scattering is normally weak, hence why the SERS technique is preferred (Das & Agrawal, 2011). This technique yields information about the molecular interaction between the surfaces, allowing low concentrations of the analyte to be detected. It also increases the intensity of the Raman signal up to 10^4 to 10^6 fold enabling faster and higher accuracy detection of samples (Zeisel et al., 1998). SERS has previously been used to detect and identify explosives, narcotics in trace amounts and molecules of biological interest (Andreou et al., 2013).

SERS allows an enhancement in the Raman signal due to the surface plasmons of silver or gold being excited resulting in an increase in the electric field that surrounds the metal (Andreou et al., 2013). A SERS colloid is a system where finely divided particles remain dispersed in a continuous matrix (White & Hjortkjaer, 2014). Smooth nanoparticle spheres do not offer a significant enhancement, these nanoparticles need to be aggregated to generate 'hot spots' where the enhancement increases significantly. For example, the addition of chloride or nitrate ions leads to aggregation which increases the SERS signal. Therefore the key to obtaining an optimised SERS method depends on the concentration of the colloid along with the concentration of an aggregating agent. The role of the aggregating agent is to create aggregates that generate 'hot spots' which is where you get the surface enhancement. Chen et al (2010), used sodium chloride as an aggregating agent to analyse caffeine using SERS. Other aggregating agents have been used in the literature for example, sodium borohydride (Creighton, 1979) and hydroxylamine phosphate (White, 2014).

One of the main limitations of developing a colloidal system is the ability to produce a colloid that has good batch-to-batch reproducibility and good stability (White & Hjortkjaer, 2014). To make this technique acceptable in real-world applications it needs to be:

- Reproducible with a minimum of 10% variation between substrates and laboratories.
- Stable for at least 8 months from fabrication
- Provide an enhancement factor of at least 10^6
- Acceptable cost

There are a number of different synthetic routes that are taken to develop colloidal systems with the preference being the use of silver colloids. There are different methods that have been used to develop and produce silver colloids (White & Hjortkjaer, 2014). Creighton developed a method using reduced silver nitrate with sodium borohydride however the stability of the colloid caused problems. Another method that has been used is a silver nitrate system using sodium citrate as the reducing agent (Lee and Meisel, 1982). Their method has been used extensively but the data present indicated that there were problems with reproducibility of batches both within and between labs. A number of attempts have been made to stabilise a silver colloidal system with the use of sodium citrate to produce reproducible colloids nevertheless more problems have been established. Kier, et al (2002) used borohydride reduction of silver nitrate with the addition of trisodium citrate, they found that when observing the UV spectral data it indicated that there was a wide range of particle size. The interaction between the analyte and the silver surface is complex and the addition of stabilising ions can be undesirable as this could modify the particle surface and can contribute to difficulties in the aggregating process which is a key step in the SERS procedure (White & Hjortkjaer, 2014).

When a colloid is produced the silver metal particles have a charge on their surface which keeps them dispersed in a medium (White & Hjortkjaer, 2014). The reason these colloids become unstable is because they lose their charge over a period of time, resulting in the colloid becoming unstable and the metal particles dropping out of solution. A stable silver colloid with strong scattering has been produced by reducing silver nitrate with hydroxylamine phosphate (White & Hjortkjaer, 2014). The rapid introduction of the silver nitrate solution to the reducing agent is a critical factor in producing good batch-to-batch reproducibility. This method has been used for over two years for the SERS detection of fraudulent use of fuels which is a further testimony to the reproducibility and long shelf life of the colloid. As this method only uses very small quantities of inexpensive reagents confirming that this HPAg colloid meets all of the requirements that are stated above.

Chen et al (2010) found that when analysing caffeine using SERS there was better enhancement when using the silver colloid and the aggregating agent (NaCl) in comparison to when the colloid was used on its own. They found that when using low concentrations of

the silver colloid on its own the Raman bands are not evident until it reaches a maximum concentration of $1.75x10^{-4}$ M. This was also seen when analysing caffeine with the borohydride-reduced silver colloid and the NaCl aggregating agent. They were successfully able to analyse caffeine and could conclude that the concentration of the colloid and the concentration of the aggregating agent plays an important role in SERS spectroscopy.

There has been an increase in the number of drug-related cases in emergency rooms appearing globally. In 2009, America found that the number of these cases (4.6 million) almost doubled that in 2004 (2.4 million) (Farquharson et al., 2011). In order to determine drug abuse and drug overdose screening and confirmation techniques are required (Farquharson et al., 2011). Immunoassay kits are used for screening by providing drug identification, however they are subject to a number of interferences leading to false positives (Farquharson et al., 2011). The use of GC-MS is commonly looked on to provide the confirmation and quantification of the drugs in question. However routine identification can be time-consuming (Schauer et al., 2005) which will in turn delay diagnosis. In order to meet the increased number of drug cases in emergency rooms Farquharson et al (2011) have investigated the ability of using SERS to detect and identify a number of drugs in saliva.

Farquharson et al (2011) analysed 152 drugs ranging from illicit drugs, prescription, over-the-counter and their metabolites using SERS with fused gold colloids. They successfully developed a method for measuring drugs in saliva by combining solid-phase extraction to separate the drugs and then used a SERS-active capillary to conduct the final analysis. Their method allowed the detection and identification of cocaine (50 ng/mL), PCP (1 μg/mL), diazepam (1 μg/mL) and acetaminophen (10 μg/mL). The analysis was performed in less than 10 minutes.

Farquharson et al (2005) have also analysed the detection of 5-fluorouracil in saliva using SERS. This study was conducted because being able to determine the dosage of chemotherapy drugs remains a challenge in the treatment of cancer due to overdosing. Being able to identify different drugs in saliva is increasingly important as it will enable testing that can be done quickly and effectively to determine what substances have been taken especially when someone is suspected to be under the influence whilst driving.

Current clinical lab analysis of drugs in overdose patients involve extensive sample extraction and GC-MS analysis which can take as much as one hour to perform as previously stated. In order to overcome this limitation Shende et al (2005), investigated metal-doped sol-gels to separate the drugs and their metabolites from saliva and then generate SERS spectra. This allowed them to analyse cocaine, its metabolite benzoylecgonine, and several barbiturates in concentrations of μg/mL.

Andreou et al (2013) conducted a study into detecting drugs of abuse namely methamphetamine in saliva using SERS and microfluidics. However methamphetamine has a relatively low affinity for silver and so in order for SERS detection to be possible methamphetamine was chemically modified to enable it to bind to the substrate. A microfluidic device was developed that enabled the transportation and aggregation of SERS active silver nanoparticles that aided in the detection of methamphetamine in saliva. Their results provide evidence for a rapid detection method that can be used to identify narcotics in biological fluids (saliva) which can potentially be used in other applications such as toxin detection and groundwater analysis.

Pavel et al (2002) looked at the analysis of caffeine at different pH values using a sodium citrate reduced Ag colloid which was used along with a NaCl solution to aggregate the colloid dispersion. They concluded that the flat orientation of caffeine attached to the Ag through the electrons and the lone pair of the N9 atom for neutral and basic values. When the caffeine was in at an acid pH value it was absorbed onto the Ag through one or both of the oxygen atoms. When caffeine was neutral or basic it absorbed onto the Ag surface through the electrons and the lone pair of the nitrogen atoms. When they compared the SERS spectrum of caffeine on Ag sol (pH 2.5) to the FT-Raman spectrum of solid anhydrous caffeine they noticed small shifts in the bands. They concluded that both electromagnetic and chemical mechanisms are involved in the interaction of caffeine and the Ag colloid due to large differences in the relative intensities of some of the bands, in particular carbonyl stretching. However, the change in the SERS pattern indicates that the electromagnetic mechanism is the dominant one.

2.6 Quantitative methods

Using SERS for quantitative analysis remains a challenge because of (a) difficulties associated with the production of reproducible SERS active substances, (b) the complex spatial and temporal characteristics of SERS substrates, (c) the strong dependence of the SERS enhancement of the distance between the analyte and the SERS substrate, and (d) variations of SERS enhancement with the surface coverage of the analyte on the substrate (Zhang et al., 2005). Quantitative concentration analysis using SERS must contend with variations that are produced by changes in excitation. In order to correct for these variations an internal or external standard is used to calibrate the correlation. Zhang (2005) describes a new isotopically edited internal standard (IEIS) that enables quantitative SERS measurements over a wide range in concentration with accuracy and reproducibility. They use deuterated rhodamine 6G (R6G-d4) as an internal standard to analyse rhodamine 6G (R6G) with a silver colloid. They found that some of the Raman bands of R6G-d4 shifted with regards to their location in R6G. This is consistent with the mass of deuterium being higher in comparison to hydrogen. They concluded that their method has shown to be reliable and reproducible.

One of the draw backs for using SERS as a quantitative method is that there can be variability so an internal standard would be used. An internal standard (IS) is a chemical substance that is added to a standard/sample improving the precision of quantitative analysis. An IS is a known concentration of a substance that is present in every sample that is analysed. The IS then can be used for calibration by plotting the ratio of the analyte to the internal standard therefore aiding quantitation. An internal standard behaves similarly to the analyte being analysed but is able to provide a signal that can be distinguished from the analyte. Ideally, factors that affect the signal of the analyte will affect the signal of the IS. Therefore the ratio of the signal from the analyte and IS will exhibit less variability than the analyte signal alone.

Katainen E (2007) looked at quantifying amphetamine in seized street samples using Raman spectroscopy. They used sodium dihydrogen phosphate as their internal standard and were able to quantify ten independent amphetamine seizures. Sodium dihydrogen phosphate

was used as an IS because it possesses a characteristic band at 880 cm^{-1}. When looking at the amphetamine spectrum it has three bands between 1050-950 cm^{-1} and a signal free area around 880 cm^{-1}. They concluded that Raman spectroscopy appears to be a potential candidate for quantitation of illicit drugs and adulterants.

Alvi et al (2011) and Perera et al (2010) both use internal standards when analysing caffeine using the HPLC. Alvi et al (2001) used an internal standard, antipyrine for their analysis when they compared the peak area of caffeine and the IS to determine the quantitation using a calibration curve. Perera et al (2010) used benzotriazole as there IS. They again used a ratio of the peak height of caffeine and benzotriazole in order to determine the concentration of unknown samples (Perera et al., 2010).

2.7 Oral fluid

The two main biological fluids used to detect drugs are blood and urine; however the methods to obtain these fluids are invasive. Substances can be detected in oral fluid for a short period of time, normally 12-24 hours after the consumption. Therefore using oral fluid for detecting recent drug use is suitable especially for clinical toxicology and for roadside testing (Bosker & Huestis, 2009). Oral fluid is produced by a number of glands and consists of 99.5% water and trace amounts of proteins. The fact saliva is mainly water makes it easy to analyse (Farquharson et al., 2011). Saliva is increasingly being used as an alternative in pharmacokinetic studies, therapeutic drug monitoring and investigating the use of illicit drugs. Particular interest has been expressed by law enforcement agencies for road side testing of potentially intoxicated drivers (Kintz & Samyn, 1999). In 2012 the UK government announced a new drug driving offence; if specific controlled drugs are in the body over a certain acceptance limit regardless whether they are impaired they will be committing an offence. In 2015 8 prescribed drugs and 8 illicit drugs were added into the new regulations, amphetamine was added April 2015. The Home Office were developing a roadside screening device based on SERS that is capable of analysing drugs in oral fluid for the past 10 years, however this is no longer a Home Office project (Butcher, 2015). There are currently two drugs that are analysed at the roadside; cocaine and cannabis and the test is by Securetec. Securetec provides products for the detection of addictive and dangerous substances; one of their products is DrugWipe® S. It has been developed for roadside testing of cannabis and

cocaine. The detection limit of the active substance Δ9-THC in saliva is 10 ng/ml. It is claimed to be one of the best drug tests in the world with low sampling volume.

The main limitations of using saliva to analyse the presence of drugs is that the amount of matrix collected is smaller in comparison to urine and the level of drugs in urine is a lot higher than in saliva; due to the drugs being concentrated by the kidneys (Kidwell et al., 1998). However due to the advances in analytical instrumentation all the requirements for forensic testing can be met with saliva as well as urine and blood. Oral fluid has been found to be suitable for the detection of drugs that have been recently taken and is easily available for collection (Lund et al., 2011). Another advantage of using oral fluid to detect drugs is that the samples can be collected under close supervision to prevent substitution or adulteration, which is a problem when sampling urine (Øiestad et al., 2007).

Table 3: Different analytical techniques that have detected drugs in oral fluid

	Drug(s) detected in oral fluid	Analytical technique	Extraction Technique	Internal Standard (examples)
Øiestad et al (2007)	Amphetamine, Cocaine, Codeine, Diazepam, LSD, MDA, MDMA, Methamphetamine, Methadone, Morphine, THC, Zopiclone	LC-MS-MS	None	Deuterated Internal Standards Morphine-d3, Amphetamine-d3, Methamphetamine-d11, MDMA-d5, THC-d6
Badawi et al (2009)	Morphine, Amphetamine, MDA, MDMA, Methamphetamine, 6-Acetylmorphine, 7-Aminonitrazepam, Tramadol, Cocaine, Clonazepam	UPLC	SPE	Deuterated Internal Standards
Gunnar et al (2005)	THC, Diazeopam, Cocaine, Methadone, Codeine, Oxazepam	GC-MS	SPE	Deuterated analogues THC-d3, Diazeopam-d5, Cocaine-d3, Methadone-d9
Alvi et al (2011)	Caffeine	HPLC	None	Antipyrine
Perera et al (2010)	Caffeine, Paraxanthine	HPLC	LLE	Benzotriazole

Different analytical techniques for analysing drugs of abuse in saliva are being explored. Table 3 summarises the different techniques and the drugs detected. Liquid chromatography–tandem mass spectrometry (LC-MS/MS) was used by Øiestad et al (2007),

they found that the sample preparation was easy, the method required no derivatisation and there was only a short analysis time. However several of the components co-eluted and could not be differentiated. Lund et al (2009) and Badawi et al (2009), found that when switching to ultra-performance liquid chromatography (UPLC) the run time was shorter and satisfactory separation was achieved. Only a limited amount of oral fluid is available for drug analysis, therefore it is crucial to have a multicomponent method usually with a low limit of detection. Gunnar et al (2005), reported a procedure for the identification, screening and quantitation of 30 drugs of abuse in saliva using GC-MS with solid-phase extraction (SPE) and derivatisation. Wood et al (2005) and Mortier et al (2002) both validated a method for the detection of the most prevalent illicit drugs and their metabolites in saliva using LC-MS/MS; however neither THC nor benzodiazepines were detected using these methods.

Wylie et al (2005) used GC-MS and LC-MS-MS to detect 49 licit and illicit drugs in oral fluid. This provided a method that was sensitive and selective for the analysis of these drugs. Initially Δ^9-THC was included in the analysis, but a white residue was obtained in the fraction containing Δ^9-THC which caused interference. Therefore this method was unable to analyse Δ^9-THC.

Wood et al (2005), provided a study looking into quantification of amphetamine in oral fluid using LC-MS/MS. They found that using the traditional confirmatory assay (GC-MS) was problematic due to the limited amount of sample. They produced a method that was simple and rapid which exploits the specificity of tandem spectrometry in order to obtain a sensitive assay. This allowed the quantification of 6 commonly abused amphetamines in oral fluid by using a clean-up method followed by LC-MS/MS. This developed method provided sensitivity, speed, simplicity and reliability over the traditional GC-MS method. This LC-MS/MS method was able to detect and quantify amphetamines found in oral fluids that had been collected from people suspected of driving under the influence of drugs which were previously undetected by GC-MS.

It can be seen in the literature that a lot of research is going into the analysis of drugs in oral fluid and the ability to detect and quantify. When looking at detecting caffeine in saliva a lot

of previous research has gone into developing a method with the use of an extraction technique.

2.8 Liquid-liquid extraction

There are different solvents for liquid-liquid extraction that will successfully extract caffeine from various sources. The most commonly used solvents are chloroform, methyl chloride, ethyl acetate and carbon dioxide (Mumin et al., 2006). Mumin et al, (2006) successfully extracted caffeine from green tea, black tea and coffee using a liquid-liquid extraction and interferences were removed by employing solid-phase extraction. The absorbed caffeine was then eluted twice with 2 mL of chloroform and dried. They were then analysed on the HPLC for quantitation and were able to quantify the caffeine.

Shende et al (2005) developed a SERS method for the analysis of several barbiturates in saliva. They analysed the saliva samples directly and found that the resultant spectra were very similar and concluded that a chemical separation might be required to extract the drug from saliva. Shende et al (2005) continued to develop a method that separated drugs from saliva. When analysing caffeine, phenobarbital and cocaine they found that adding 1 M sulphuric or acetic acid, heat and sonication successfully separated the drugs from saliva. They also concluded that this extraction technique was sufficient enough to degrade the saliva proteins allowing the drug in question to bind to the SERS-active metal surfaces without any interference from the proteins.

Øiestad et al (2006) used a LLE technique to analyse drugs in oral fluid. Their samples were extracted by adding a ethylacetate:heptane solution, mixing for 10 minutes and the centrifuging. This enabled them to analyse a large number of drugs including low-dose benzodiazepines. Perera et al (2010) also used an extraction technique to extract caffeine from saliva. The solvent that was used in this study was ethyl acetate, the samples were mixed for 5 minutes and then centrifuged.

2.9 Summary

As can be seen by the literature there are various methods that can be used to analyse different legal high substances and a lot of these legal high substances contain the

adulterant caffeine. The use of Surface-Enhanced Raman Spectroscopy is one that has recently started drawing interest with regards to analysing drugs due to its low limit of detection. Saliva testing for drugs is a developing area and that SERS offers the potential to provide a rapid portable analysis. This work will focus on caffeine and metabolites as model compounds to develop a procedure but once developed the technique can be applied to other drugs including NPS. SERS will be looked at within this study to analyse caffeine found in oral fluid.

2.9.1 Aims and Objectives

The main aim of this project is to provide an analytical method for the analysis of caffeine in oral fluid. This will be done by using Surface-Enhanced Raman Spectroscopy as the screening technique coupled with HPLC-DAD as a confirmation technique. A SERS method will be developed, optimised and validated for the analysis of caffeine in methanol, looking at different instrumental parameters eg filter, acquisition time and hole. In addition the parameters around the concentration of the silver colloid and aggregating agent will be investigated. This established method will be applied to caffeine in water and subsequently caffeine in saliva. An optimised HPLC method will be developed and validated for the quantification of caffeine. These methods will then be applied to the analysis of caffeine in oral fluid.

3. Materials and Methods

3.1 Instrumentation

3.1.1 High-performance liquid chromatography (HPLC)

The chromatographic system used was Merck Hitachi LaChrom diode-array detector L-7455 with an Interface D-700, LaChrom column oven L-7350 and a solvent degasser L-7612. The detector wavelength used was 245 nm. A Zorbax ODS C18 column (25 cm x 4.5 mm x 5 µm) was used for the separation of the analytes. The mobile phase used was methanol.

3.1.2 Raman Spectroscopy

The system used was a Horiba Jobin Yvon Labram. A 532 nm green laser was used as Raman excitation, using these settings: filter 1%, hole 1000 µm, slit 100 µm and scan range of 200-1700 cm^{-1}. For each Raman scan, the acquisition time was 20s and 2 scan cycles (unless stated otherwise). The microscope was used for powders and the cuvette attachment was used for solutions using glass cuvettes.

The software KnowItAll which is used for interpretation, identification, verification and classification of spectra was used to interpret the spectrum

3.1.3 UV-Vis

The absorption spectrum was recorded using a Shimadzu UV-Vis spectrophotometer UV-1650PC over a wavelength range of 300-700 nm using a medium scan speed and the light source at 295 nm and plastic cuvettes.

3.2 Chemicals and sample preparation

3.2.1 Chemicals

Anhydrous caffeine powder was supplied by Sigma Chemical co. The silver nitrate ($AgNO_3$), sodium hydroxide (NaOH) hydroxylamine phosphate, reducing solution (Na_2SO_3 and KCNS), dilute phosphoric acid, and potassium permanganate were used directly without any purification. All the solutions were prepared in deionised water (15 MΩ) and the caffeine was prepared in methanol. All samples and solvents were acquired from Sigma Aldrich at HPLC grade with respect to the solvents.

3.2.2 Colloid preparation

The sodium hydroxide (NaOH) was made by adding 1.335 g of NaOH to 100 mL of water. This was then diluted down to a 1 in 100^{th} dilution to make a concentration of $3.300x10^{-3}$ M (working solution) which was freshly made. Silver nitrate ($AgNO_3$) was made by adding 140.3 mg to a volumetric flask and 100 mL of water was added to give a concentration of 1.000×10^{-2} M. 20.4 mg of hydroxylamine phosphate was added to a vial and then topped up with 4 mL of water to give a concentration of 2.54×10^{-5} M.

The silver colloid for the surface enhanced Raman spectroscopy (SERS) was prepared by adding 4.5 mL of the working NaOH solution to a vial. 100 µl of Hydroxylamine phosphate was then added to the vial and rotated slowly for a few seconds. 500 µl of a $1x10^{-2}$ M $AgNO_3$ solution was added quickly and again rotated slowly for a couple of minutes. This method was based on the work of White and Hjortkjaer (2014).

UV-Vis spectrometry was used to determine the maturity of the colloid. The UV-Vis spectra were obtained by pipetting 0.06 mL of the colloid into a 4 mL disposable polystyrene cuvette and 3 mL of water was added.

3.2.3 SERS method

The colloid was then left for a month in order for it to mature. The aggregating agent sodium chloride (NaCl) was used directly without any purification and was 0.1 M concentration. 100 µl of the sample was added to the cuvette followed by 100 µl of the aggregating agent and the sample was inverted a couple times. Then 500 µl of the colloid was added and inverted again. The sample was then analysed on the Raman immediately using the cuvette attachment unless stated otherwise. All spectra were baseline corrected and 4 replicates were made.

3.2.4 Preparation of solutions used for Liquid-Liquid Extraction

The reducing solution (Na_2SO_3 and KCNS) was made by adding 5 g of Na_2SO_3 and 5 g KCNS to 100 mL of water. Phosphoric acid was made by diluting 15 mL syrupy phosphoric acid to 85

ml of water. 25 g of sodium hydroxide was added to 75 mL of water. 1.5% potassium permanganate was made by adding 0.75 g with 49.25 mL of water.

3.2.5 Liquid-Liquid Extraction procedure

1 mL of sample was added to a separating funnel and 500 µL of potassium permanganate was added. The separating funnel was inverted for 1 minute. Then 1 mL of the reducing solution (Na_2SO_3 and KCNS) was added and inverted. 100 µL of diluted phosphoric acid and 100 µL sodium hydroxide was added to the funnel and inverted. 5 mL of chloroform was added to the separating funnel, inverted for 5 minutes and left for the layers to separate for 20 minutes. After 20 minutes the bottom chloroform layer was filtered through fluted filter paper and into a vial. The funnel stem was rinsed with 300 µL of chloroform and drained through the filter paper. The filtered paper was then washed with 300 µL of chloroform. The aqueous layer was then re-extracted with 4 mL of chloroform. Both the extractions were added together, blown down to dryness using nitrogen and reconstituted in 1 mL of methanol.

4. Results and Discussion

4.1 Analysis of caffeine using Raman Spectroscopy

One of the advantages of using Raman spectroscopy is its ability to detect the presence of substances in aqueous solutions. It provides a finger print region which can be used to identify and quantify different substances. Figure 2 shows the Raman spectrum of caffeine powder along with the structure of caffeine. Caffeine has two rings that are fused these are; pyridine ring and the imidazole ring. On the pyridine ring two amide groups and two tertiary amine groups can be found. Whereas, the imidazole ring has one amide group, one tertiary group and an alkene group.

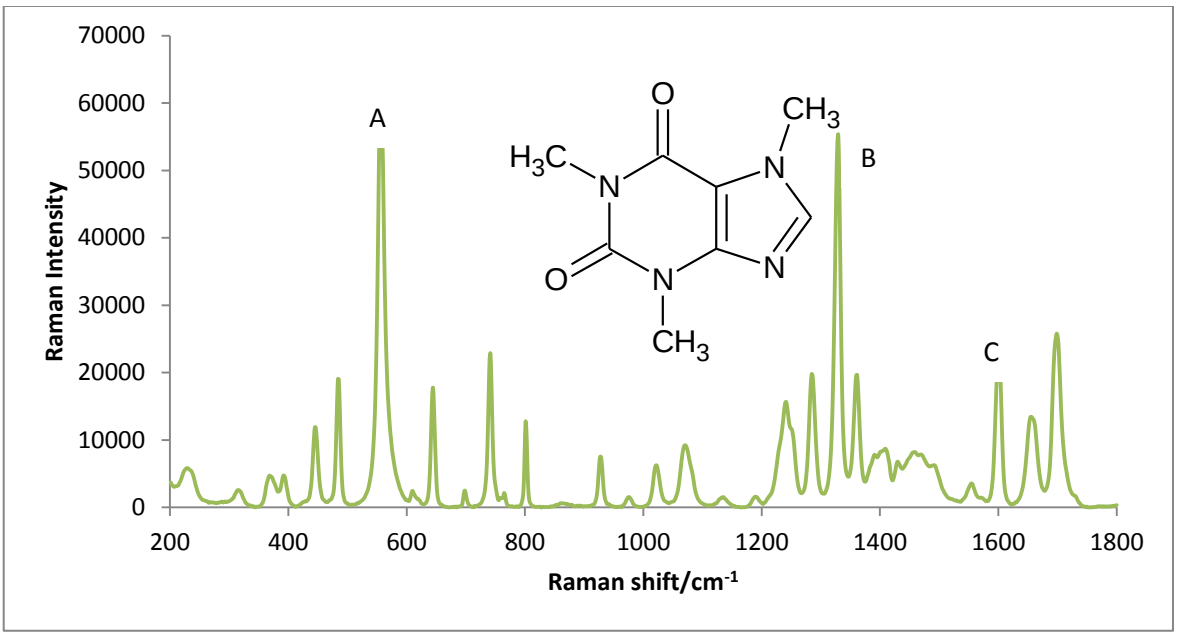


Figure 2: Raman spectrum of caffeine powder using 100% filter, 300 µm hole, acquisition time of 10 seconds and 2 scan cycles. A) 553 cm^{-1} B) 1325 cm^{-1} C) 1600 cm^{-1} . Baseline corrected

Table 4 shows the different peaks in the Raman spectrum and their assignment. There are three key bands that can be used to identify caffeine at 553 cm^{-1}, 1325 cm^{-1} and 1600 cm^{-1} which can be assigned to the pyridine ring, imidazole trigonal ring stretching and C=C stretch respectively.

Table 4: Assignment of the caffeine peaks (figure 2) using Chen's (2010) assignment table

Raman cm^{-1}	**Assignment of Caffeine Powder**	**Assignment of Caffeine in Solution**
441m	N-C-C	
481ms	C-N-C	
553vs	O=C-N or pyridine ring	O=C-N or pyridine ring
609vw	O=C-N	
641ms	O=C-N	
696w	C=O	
738m	O=C-C	
798m	N-C-H	
926m	Imidazole ring	
973w	Pyridine ring	
1020m	N-CH_3 stretch	
1067m	In plane C-C	
1130vw	C-N	
1187vw	C=N stretch	
1238m	C-N stretch	
1283m	C-N stretch	
1325vs	Imidazole trigonal ring stretching	Imidazole trigonal ring stretching
1404w	C-N antisymmetric stretching	
1450vw	C=N stretch	
1551vw	C-C stretch	
1600m	C=C stretch	C=C stretch
1657mw	C=O stretch	
1698ms	C=O stretch	C=O stretch

vw – very weak, w – weak, m – medium, ms – medium strong s –strong, vs – very strong

Figure 3 shows the Raman spectrum of caffeine in an aqueous solution. It demonstrates that Raman spectroscopy is capable of analysing aqueous solutions.

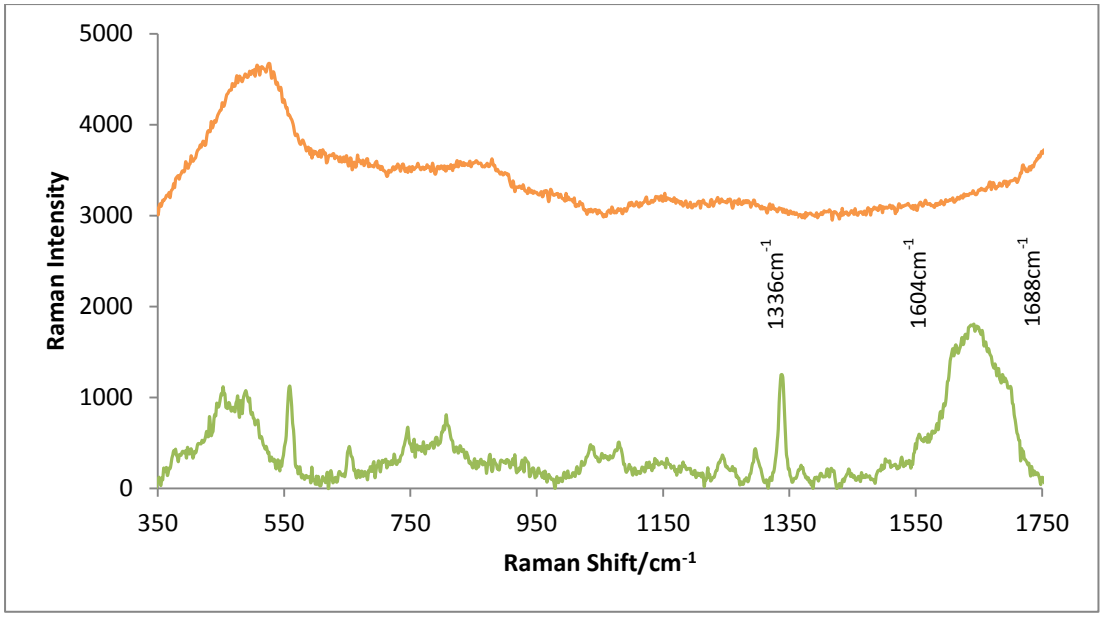


Figure 3: Raman spectra of 10 mg/mL caffeine in water (green) and spectra of blank water (orange) using 100% filter, 1000 µm hole, acquisition time of 10 seconds and a scan cycle of 2. Baseline corrected

The spectrum of the caffeine powder shows similarities to the spectrum of the caffeine in aqueous solution. There is a strong peak that can be seen in Figure 2, table 4 at 1336 cm^{-1} which can be assigned to the imidazole trigonal ring stretching. Some other peaks that can be used to distinguish caffeine are the two at 1604 cm^{-1} and 1688 cm^{-1} which are responsible for the vibrational bands of C=C stretch and C=O stretch respectively. The spectrum of caffeine in water is weaker which can be seen by the lower intensities and the baseline appears to be nosier. A glass cuvette was used to analyse caffeine in water, figure 3 demonstrates that there is no interference from the glass. Figure 3 also shows that the additional peaks in the caffeine and water spectrum are due to the caffeine and not the water. This is confirmed by comparing a water blank where it is evident that there are no peaks present. Raman spectroscopy can be used to quantify solutions and therefore be used to determine the concentration of an unknown sample.

4.2 Quantitative Raman spectroscopy

A calibration curve was produced by using the peak height of caffeine found at 1336 cm^{-1} against the concentration of known standards. The results are shown in table 5 and figure 4.

Table 5: Calibration results for the different caffeine standards

Concentration (mg/mL)	Mean Peak height of Caffeine (n=8)	Standard Deviation
1	251.04	25.18
3	650.26	174.10
5	1002.84	418.01
7	1732.92	88.59
10	2222.54	536.74

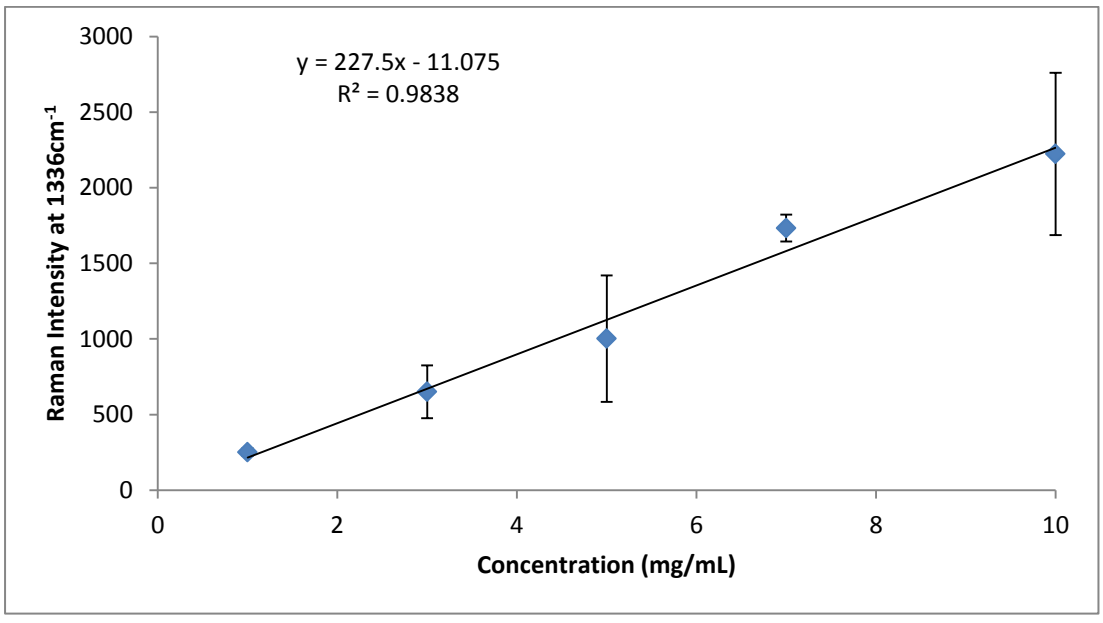


Figure 4: Calibration results of the different caffeine standards showing the calibration equation (n=8, error bars are ±1SD)

The Raman signal at 1336 cm^{-1} increases with the increasing concentration of the solution. This demonstrates that the Raman signal is directly proportional to the number of molecules

in solution over the concentration range. Therefore it is possible to develop a quantitative method.

4.3 Analysis of solid caffeine tablets using Raman spectroscopy

Caffeine tablets were provided to test the quantitative method by identifying whether caffeine is present and quantify the caffeine concentration. One tablet was dissolved in an aqueous solution and analysed using the Raman method. The solubility of caffeine in water at room temperature is 2g / 100mL. The caffeine tablet contains 200 mg of caffeine; it was dissolved in 10 mL of water and put in the sonic bath for 10 minutes to help dissolve the caffeine fully. Figure 5 shows the spectrum of the caffeine tablet.

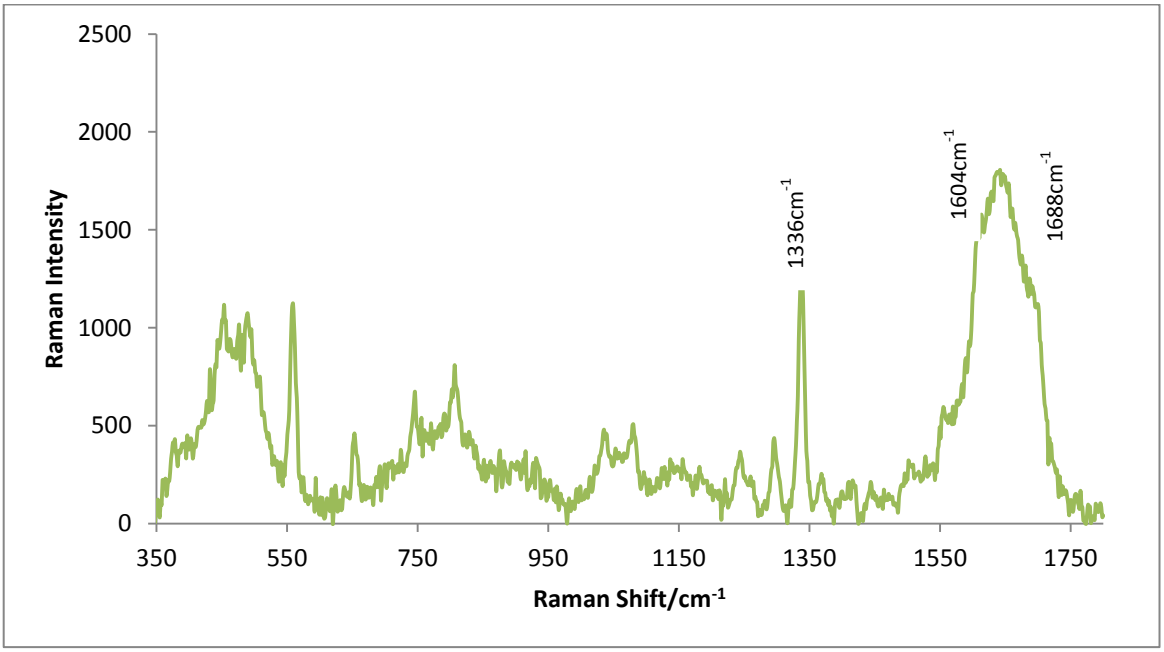


Figure 5: Caffeine tablet in water using 100% filter, 1000 hole, acquisition time of 10 seconds and a scan cycle of 2. Baseline corrected

Comparing figure 3 (10 mg/mL spectrum) and figure 5 (above) it is evident that caffeine is present as it has the same peak at 1336 cm^{-1} that is present in the caffeine standards (figure 3). It also has other caffeine peaks present, the two peaks found in the caffeine standard at 1604 cm^{-1} and 1688 cm^{-1} can be seen in the caffeine tablet but the peaks are not separated. 1336 cm^{-1} is a good band to use as it remains sharp. Table 6 shows the different correlation

coefficients when the unknown caffeine tablet was compared to the 5 caffeine standards used for the calibration curve. Looking at the correlation coefficient it shows that the unknown caffeine tablet has a correlation coefficient of 0.996 when compared to the caffeine standards. The correlation coefficients were obtained by exporting the spectra into Excel and using the correlation coefficient function in Excel. This suggests that the unknown caffeine spectrum has the same pattern of peaks that the standards have. Whatever else might be in the tablet isn't interfering with the caffeine spectrum as there is good correlation match to the standards.

Table 6: Correlation coefficient of the unknown caffeine tablet (U) compared to the standards of known concentration.

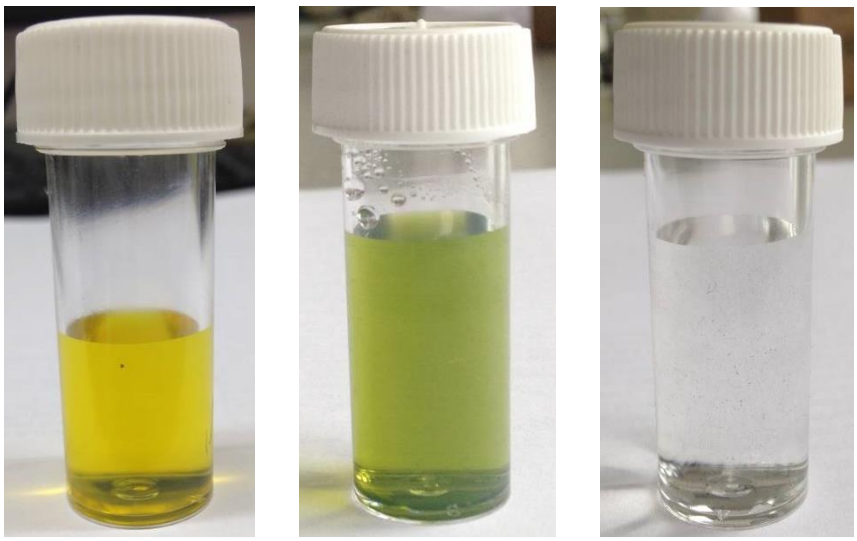
U compared to standards (mg/mL)	Correlation coefficient
U:10	0.974
U:7	0.983
U:5	0.988
U:3	0.996
U:1	0.995

By looking at the lower concentrations of caffeine (1 mg/mL) using Raman spectroscopy it may be difficult to determine if caffeine is present in a sample. Surface-Enhanced Raman Spectroscopy is capable of analysing samples at a much lower concentration. In order to use Surface-Enhanced Raman Spectroscopy, a SERS substrate is required. UV-Vis was used to characterise and establish that the colloid is being produced in a repeatable way as only small volumes were prepared at a time.

4.4 Preparation of the silver colloid

Using a method developed by Peter White (2014), silver colloids were produced using the method stated previously (section 3.2.2) In order to produce a stable colloid the addition of the silver nitrate solution has to be done very quickly as it is a critical factor in the chemical reaction. After the addition of the silver nitrate, the colloid solution turns a dark brown

colour. Once the colloid had matured it produces a straw coloured solution (Fig 6a). The UV-Vis spectra can be used to determine different features of colloid such as the size of particles and its degree of aggregation. It has previously been established that the number of particles in the solution is proportional to the absorbance (White & Hjortkjaer, 2014). A broadening of the bandwidth indicates a wider particle size distribution. Any aggregation of a colloid is also indicated by a decrease in absorbance and the appearance of a second band at a longer wavelength this can be seen in figure 7 when looking at TP14.



A B C

Figure 6: A) photograph of a stable colloid (after 3 months), B) and C) photograph of two colloids that show different stages of a 'crashed' colloid

Within the colloid solution the silver particles have a charge on their surface; this charge is what keeps the particles dispersed. Figure 6b and 6c show images of two colloids that have become unstable at different degrees. Figure 6b had gone from being a straw colour to a green solution and figure 6c the silver particles have 'dropped out of suspension'; the particles were visible along the bottom of the vial. This shows that there is batch to batch variability and if the colloid isn't prepared in the exact same way then some of the colloids will 'crash'. One explanation for this behaviour is that the colloid has become unstable due to the loss of charge from the silver particle surface. This could be caused by two things; the

dissolution of the ion onto the aqueous solution or the migration of the silver surface ions to the container wall (White & Hjortkjaer, 2014). Looking at Figure 6c the silver particles have precipitated (crashed) which indicates that the colloid has a short shelf life.

Looking at the UV-Vis shown in figure 7 a sharp narrow peak indicates a good quality colloid with narrow nanoparticle size distribution. Table 7 shows the height of the colloid peak and its half height.

Table 7: The UV-Vis absorbance of the peak height and the peak width of 3 colloid samples and 1 diluted colloid sample

Colloid Sample	λ max (nm)	Peak width at half height (nm)
KW9	397	55
KW18	397	55
TP14	393	40
KW9 diluted 50%	397	57

The peak width shows that colloid KW9 and KW18 has matured as it shows a bandwidth of around 51 nm (White & Hjortkjaer, 2014). When looking at KW9 and KW18 the colloid is only a month old whereas TP14 is four months old suggesting it has reached its full maturity. After the initial preparation of the colloid, there is a decrease in the bandwidth. The ageing process resulted in the stabilisation of the colloid and its bandwidth. It was observed that these changes occurred more rapidly within the first 4 weeks of the colloid preparation and then started to stabilise to produce a colloid which meets the four standards that have been set. Figure 7 shows the spectra of the 1 month old colloid, 4 month old colloid and the effect of diluting the colloid by 50%.

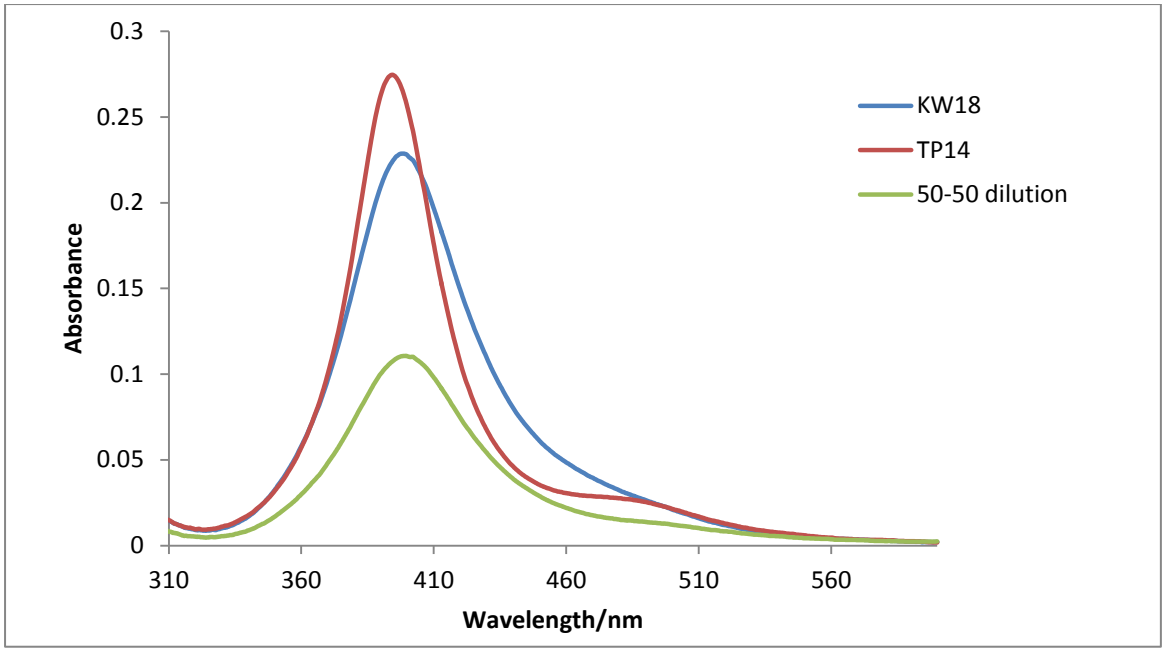


Figure 7: UV-Vis absorbance spectrum of the silver colloids: KW18 (1 month old colloid), TP14 (4 month old colloid) and the colloid diluted by 50% (KW9 1 month old)

Figure 7 demonstrates the effect of leaving the colloid for a period of time in order for it to mature. When looking at the 50% dilution it is evident that the absorbance is considerably lower than KW18 due to there being less colloid nanoparticles in the sample. Therefore there is a decrease in absorbance as fewer particles are present. When looking at TP14 the absorbance is higher as it has had a longer time to mature. TP14 also suggests that there is some aggregation as there is a hump around 480 nm. Table 8 shows the effect of diluting the colloid against the absorbance.

Table 8: Absorbance values for the diluted colloid using 295 nm wavelength

Percentage dilution (%)	Mean (n=3)	Standard Deviation
0	0	0
25	0.053	0.0040
50	0.118	0.0070
75	0.174	0.0180
90	0.197	0.0290
100	0.255	0.0102

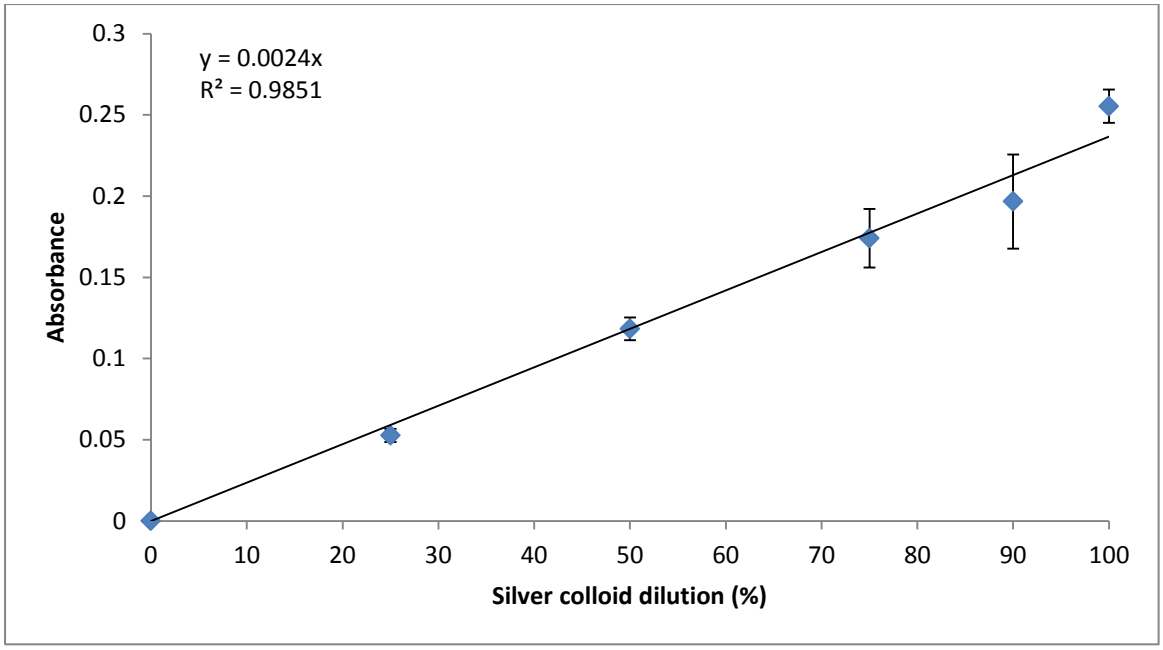


Figure 8: The relationship between the absorbance and the dilution of the colloid (n=3, error bars are ±1SD, 295 nm wavelength)

As can be seen from figure 8 the absorbance of the colloid is directly proportional to its percentage dilution. The direct proportional relationship between the absorbance and concentration which is demonstrated in Figure 8 is the Beer-Lambert law.

4.5 Optimising Surface-Enhanced Raman Spectroscopy (SERS) for the analysis of caffeine in methanol

To optimise a SERS method that is able to analyse caffeine in methanol a number of parameters can be altered. The different instrumental parameters that can be changed on the Raman spectrometer range from the filter, the hole, the acquisition time and the slit etc. The filter protects the sample from the emitted light and reduces the scattering of the laser. The hole is changed when using the confocal microscope; it allows the depth of field to be altered when looking at solids, with aqueous solutions the hole is not an important factor as it is important to collect as much of the scattered light to give a Raman signal. By changing the acquisition time it limits the amount of time the laser excites the sample for and controls the amount of time the light is collected. The longer the acquisition time the more

scattered light is collected. The slit width is crucial for the spectrometer's performance. The slit width can be found at the entrance to the monochromator which projects the image onto the detector plane. The resolution and throughput is determined by the slit width. The bigger the slit means more light is going through the monochromator, which gives it a larger bandwidth but poorer resolution.

To ensure that the signal from the SERS is due to surface enhancement some controls have to be taken into account. The controls are put in place to show that the enhancement of the signal is down to the use of the silver colloid and the aggregating agent rather than a conventional Raman spectra of caffeine. Figure 9 shows the different controls that were analysed.

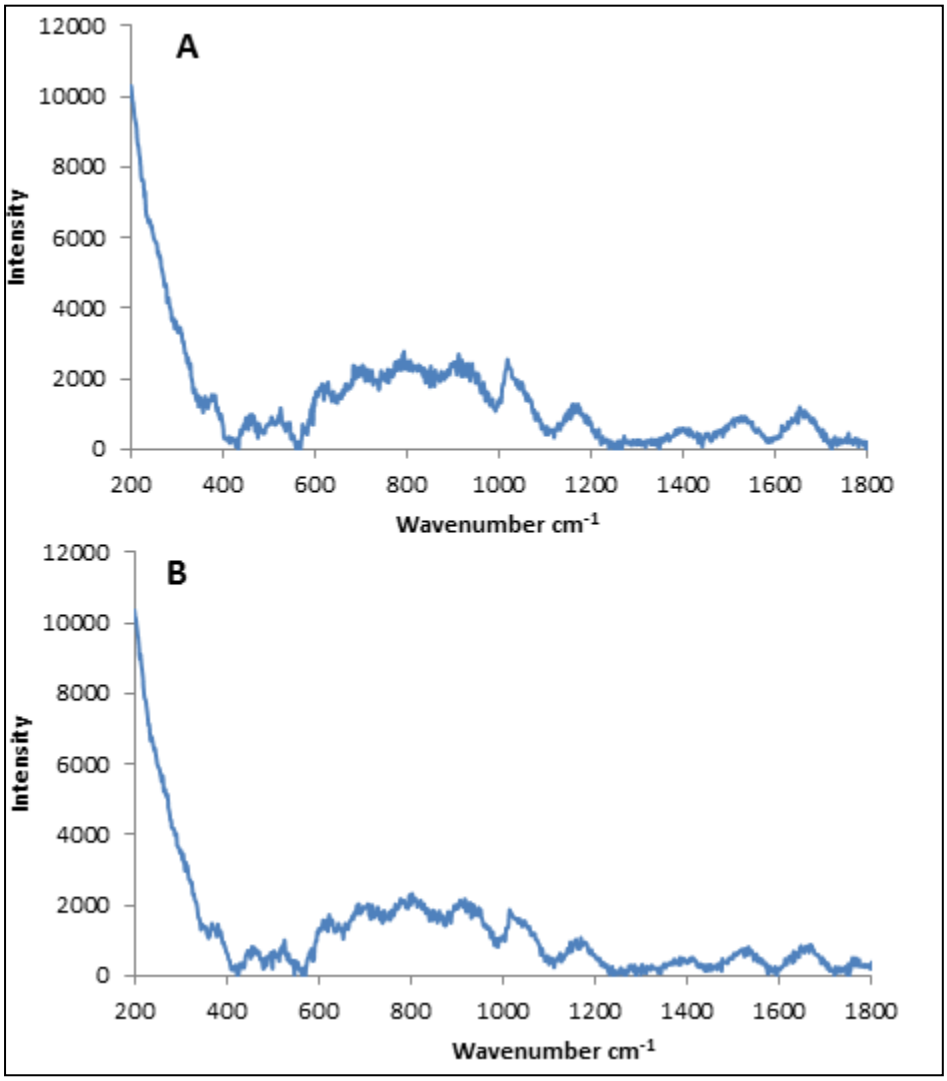


Figure 9: (A) Raman spectrum of caffeine (25 µg/mL) when added to 600 µl of water (B) Raman spectrum of caffeine (25 µg/mL) when added to 500 µl of water and 100 µl of the aggregating agent (0.1 M NaCl). Baseline corrected

As can be seen by figure 9 it is evident that there are no peaks when trying to analyse caffeine. The limit of detection of caffeine in water is not suitable for the levels of caffeine that can be found in saliva. Caffeine cannot be detected at these concentrations using normal Raman. Figure 3 shows a spectrum of a water blank where there are no peaks present.

To demonstrate the effect that SERS has on the analysis of caffeine, caffeine was analysed using the Raman spectrometer along with SERS (Figure 10).

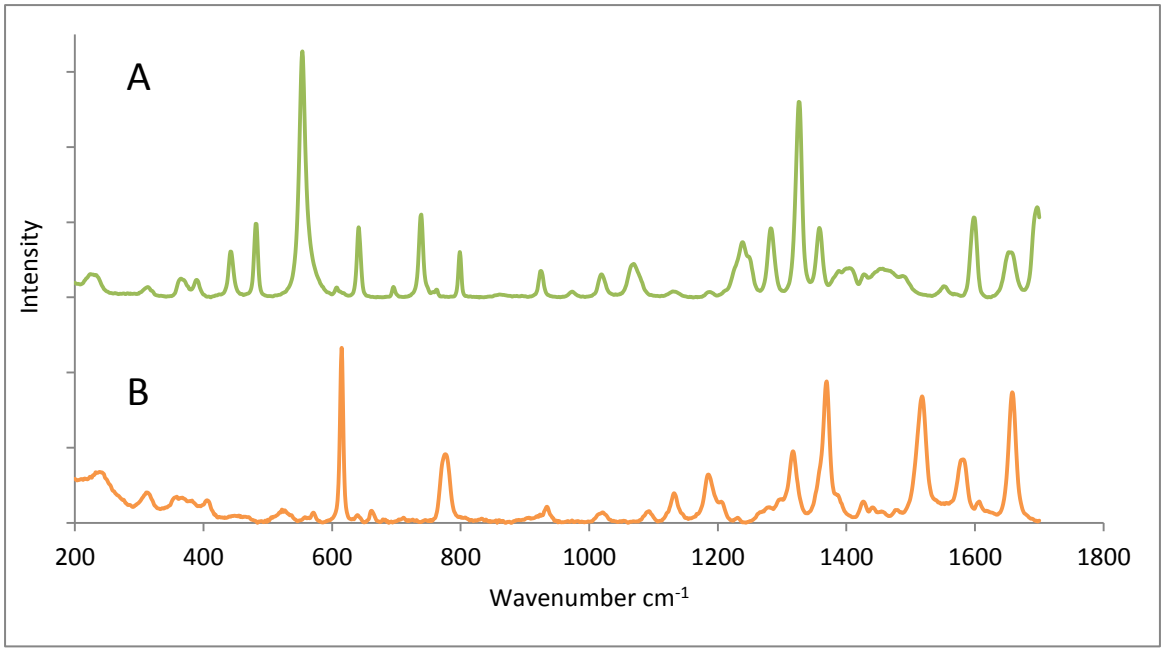


Figure 10: (A) Raman spectrum of caffeine powder. (B) SERS spectrum of 25 µg/mL caffeine with silver colloid and NaCl (0.1 M) Baseline corrected

Comparing figure 10a to figure 9 it is clear that there are peaks from caffeine present when using SERS at a level much lower than can be seen with normal Raman spectroscopy. This demonstrates that SERS is providing an enhancement compared to normal Raman.

Table 9 summarises the Raman frequencies of the conventional Raman and surface-enhanced Raman spectrum of caffeine according to Chen et al (2010).

Table 9: Peaks of caffeine with normal Raman and SERS bands for silver colloid (Chen et al, 2010).

Raman cm^{-1}	**Chen's Raman cm^{-1}**	**SERS**	**Chen's SERS**	Assignment
441m	**445m**			N-C-C
481ms	**481ms**			C-N-C
		518m	**505m**	N-C-C
553vs	**556vs**	**555vw**	**555vw**	O=C-N or pyridine ring
588vw	**609vw**			O=C-N
641ms	**642ms**			O=C-N
696w	**698w**			C=O
738mw	**742s**	**722w**	**746vw**	O=C-C
763vw	**763vw**			
798s	**800s**			N-C-H
926s	**925s**			Imidazole ring
973w	**975w**			Pyridine ring
1020m	**1020m**	**1014m**	**1014m**	N-CH_3 stretch
1067m	**1070m**	**1080vw**	**1076vw**	In plane C-C
1130vw	**1133vw**	**1126w**	**1131w**	C-N
1187vw	**1189vw**	**1171m**	**1171m**	C=N stretch
1238m	**1239m**			C-N stretch
1283m	**1283m**			C-N stretch
		1309m		C-N stretch
1325vs	**1327vs**			Imidazole trigonal ring stretching
1359s				
1404w	**1409w**	**1360s**	**1363s**	C-N symmetric stretching
		1414vw		CH_3 symmetric bending
1450vw	**1456vw**			C=N stretch
1551vw	**1551vw**	**1570m**	**1556w**	C-C stretch
1596s	**1600s**	**1616m**		C=C stretch
1657m	**1656m**	**1647s**		C=O stretch
1698ms	**1698ms**			C=O stretch

vw – very weak, w – weak, m – medium, ms – medium strong s – strong, vs – very strong

The differences between the Raman and SER spectra is not uncommon as it reflects the variations that occur between the surface interactions that enhance various vibrational modes to different extents (Shende et al., 2005). The two strong bands at 553 cm^{-1} and 1325 cm^{-1} which can be seen in figure 10 are assigned to the O=C-N or the pyridine ring and imidazole trigonal ring stretching (Chen et al., 2010) which is in agreement with Chen et al (2010) who obtained bands at 556 cm^{-1} and 1327 cm^{-1} respectively. When comparing these peaks to the SERS spectrum there are small shifts between the frequencies (555 cm^{-1} and 722 cm^{-1}). However these bands considerably weaken when analysed using SERS as shown in table 9 for both the results obtained in this work and those of Chem et al (2010). Chen et al (2010) concluded that this was due to the planes of the pyridine ring being parallel to the silver surface. The bands at 1020 cm^{-1}, attributed to the N-CH_3, and 1404 cm^{-1} (C-N stretching) in normal Raman were weaker in comparison to the peaks found at 1014 cm^{-1} and 1360 cm^{-1}. The assignment of bands using the software KnowItAll corresponds with the peaks assigned by Chen et al. The peak at 1360 cm^{-1} corresponds with a N-C-N symmetric stretching which is in agreement with the assignment from Chen at 1363 cm^{-1}. The peak at 1360 cm^{-1} on the SERS spectrum has a slight shoulder which has been matched to a methyl group. It is evident that the greatest enhancement is due to the C-N vibration which indicates that the C-N group interacts strongly with the silver surface (Chen et al., 2010) and the N-CH_3 group is also in close proximity to the colloid surface.

When looking to see what parameters produce the optimum spectrum for the analysis of caffeine, the caffeine peak at 1360 cm^{-1} will be used as it is provides a good response to SERS. This peak height along with the silver chloride peak at 200 cm^{-1} will be used to for the peak height ratio calculation. Table 10 compares the changes in parameter against their peak height ratio. It is evident that with regards to the time delay, when the sample was laid for 30 minutes it gave the best signal. This can be concluded as the peak height ratio is the highest suggesting it obtained the best signal.

Table 10: The effect on the peak height ratio when the sample had been left (normalised)

Time Delay		Colloid peak height (200cm^{-1})	Caffeine peak height (1360cm^{-1})	Caffeine: Colloid peak height ratio
Time Delay	10 min	0.371108	1.0952465	2.95
	20 min	0.3755335	1.150011	3.06
	30 min	**0.257068**	**1.107083**	**4.31**
	40 min	0.3071225	1.1473995	3.73
	50 min	0.3692225	1.1426105	3.09
	60 min	0.3808825	1.1511975	3.02

Using the optimum time the other parameters were optimised Table 11 shows these results.

Table 11: The effect on the peak height ratio when changing the filter percentage and the acquisition time (normalised)

Run	Time delay (minutes)	Filter (%)	Acquisition Time (seconds)	Colloid dilution (%)	Colloid peak height (n=3)	Caffeine peak height (n=3)	Caffeine: Colloid peak height ratio (n=3)
1	30	1	1	100	0.025388	0.068715	2.71
2	30	1	5	100	0.029638	0.083584	2.82
3	30	1	10	100	0.031528	0.095726	3.04
4	30	1	15	100	0.035727	0.113728	3.18
5	**30**	**1**	**20**	**100**	**0.030666**	**0.122867**	**4.01**
6	30	10	1	100	0.055814	0.08691	1.56
7	30	10	5	100	0.033986	0.12761	3.76
8	30	10	10	100	0.039213	0.130037	3.32
9	30	25	1	100	0.036915	0.132188	3.58
10	30	25	5	100	0.293000	1.150079	3.93

When looking at table 11 it is evident that the filter percentage corresponds with the acquisition time. Comparing the spectra of the 1% filter to the different acquisition time it is evident that the best spectrum that is produced is when using a 20 second acquisition time. This can be concluded by looking at the caffeine:colloid peak height ratio, it is evident that the ratio for the 1% filter and 20 seconds acquisition time produces the best spectrum as the ratio is high suggesting that those parameters obtain the best signal. When looking at the 10% filter, it is evident that the best acquisition time is 5 seconds again as it produces a

high ratio. However when looking at the effect of the filter and the acquisition time as a whole, it is clear that the optimised parameters to use would be run 5 (highlighted in bold) as it produces the best signal with regards to the peak height ratio. This suggests that the filter is low enough so that the sample is not being saturated but there is a long enough acquisition time for the sample to generate enough energy to produce a good spectrum. The optimised Raman settings were then used for further studies.

Table 12: Effect of changing the colloid dilution on the peak height ratio (normalised)

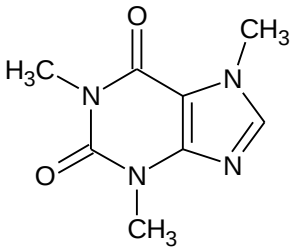
Run	Time laid (minutes)	Filter (%)	Acquisition Time (seconds)	Colloid dilution (%)	Colloid peak height (n=3)	Caffeine peak height (n=3)	Caffeine: Colloid peak height ratio (n=3)
1	30	1	20	25	0.014724	0.018561	1.26
2	30	1	20	50	0.493399	0.750212	1.52
3	30	1	20	75	0.019741	0.050471	2.55
4	**30**	**1**	**20**	**100**	**0.017205**	**0.083953**	**4.88**

Table 12 suggests that the best colloid to use is the 100%. This suggests that there is enough silver for the caffeine to bind to and in turn enables the signal to be enhanced. The other values suggest that by diluting the colloid with water essentially means that there is less silver colloid for the caffeine to bind to hence producing a weaker signal.

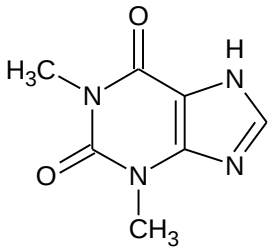
4.6 Analysis of Theophylline in methanol using SERS

The optimised method was used for the analysis of theophylline a metabolite of caffeine. Caffeine and theophylline have a very similar structure

Figure 11: Caffeine and theophylline structures



Caffeine



Theophylline

Figure 11 shows that caffeine has a CH_3 attached to all three of the nitrogen groups whereas in theophylline one of the methyl groups has been replaced by a H. When analysing the caffeine and theophylline powder using conventional Raman their spectra are very similar.

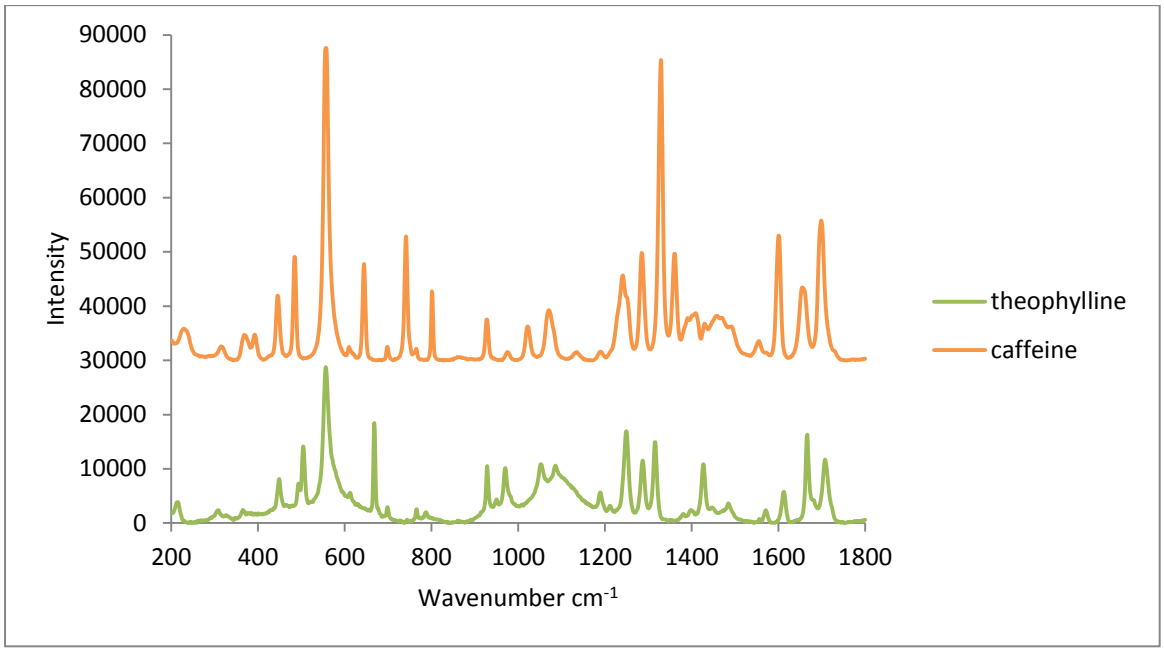


Figure 12: Comparison of Caffeine and Theophylline powder spectra using conventional Raman with 100% filter, 300 µm hole, acquisition time of 10 seconds and 2 scan cycles. Baseline corrected

Looking at figure 12 it is evident that their spectra differ which is expected, there are shifts in the bands. Table 13 summarises the different bands for caffeine and theophylline. When comparing some of the peaks there are slight shifts between each spectrum. However there are two peaks that are found in the caffeine spectrum that cannot be seen in the theophylline spectra (738 cm^{-1} and 798 cm^{-1}). This could be due to their difference in structure. Where caffeine has a N-CH_3 functional group theophylline only has NH functional group, the difference in these group could be the cause of the slight difference in their spectra. The band at 798 cm^{-1} is assigned to N-C-H which corresponds with the conclusion that this band is not present in the theophylline spectrum because of the difference in structure. The main similarity in both spectra is the band at 1020 cm^{-1} this band has been enhanced in both caffeine and theophylline; suggesting that the part of the molecule that is closest to the silver nanoparticles is the pyridine ring which confirms Chen's conclusion.

When comparing both their spectra using the correlation coefficient the value was 0.43 which suggests that the spectra differ considerably.

Assignment	Caffeine	Theophylline
N-C-C	441m	447m
C-N-C	481ms	505ms
O=C-N or pyridine ring	553vs	557vs
O=C-N	609vw	609vw
O=C-N	641ms	667s
C=O	696w	697w
O=C-C	738s	-
N-C-H	798s	-
Imidazole ring	926s	928m
Pyridine ring	973w	968m
N-CH_3 stretch	1020m	1049m
In plane C-C	1067m	1071m
C-N	1130vw	-
C=N stretch	1187vw	1190w
C-N stretch	1238m	1214s
C-N stretch	1283m	1290m
Imidazole trigonal ring stretching	1325vs	1319s
C-N antisymmetric stretching	1404w	1397w
C=N stretch	1450vw	-
C-C stretch	1551vw	1566w
C=C stretch	1600s	1608w
C=O stretch	1657m	1665s
C=O stretch	1698ms	1707m

Table 13: Peaks of caffeine and theophylline with Raman spectroscopy

vw – very weak, w – weak, m – medium, ms – medium strong s –strong, vs – very strong

When comparing the SERS spectra there are no significant differences, the only differences that can be seen is the intensity of the spectra (Figure 13). By comparing these spectra it also concludes that it is the C-N group that interacts the strongest with the silver colloid. The

difference between the two structures is bonds to hydrogen which explains why their spectra are very similar when analysing them using SERS. It would be expected to see a difference in their spectra when extending their range to 2500-3000 cm^{-1} as this is when the methyl groups are commonly found. One of the Raman bands that is present in caffeine is the band at 798 cm^{-1} which is assigned to N-C-H bond. However this band is not present in theophylline. This could be due to the hydrogen bonding between the CH_3 that is present in caffeine but has been replaced in theophylline by a hydrogen.

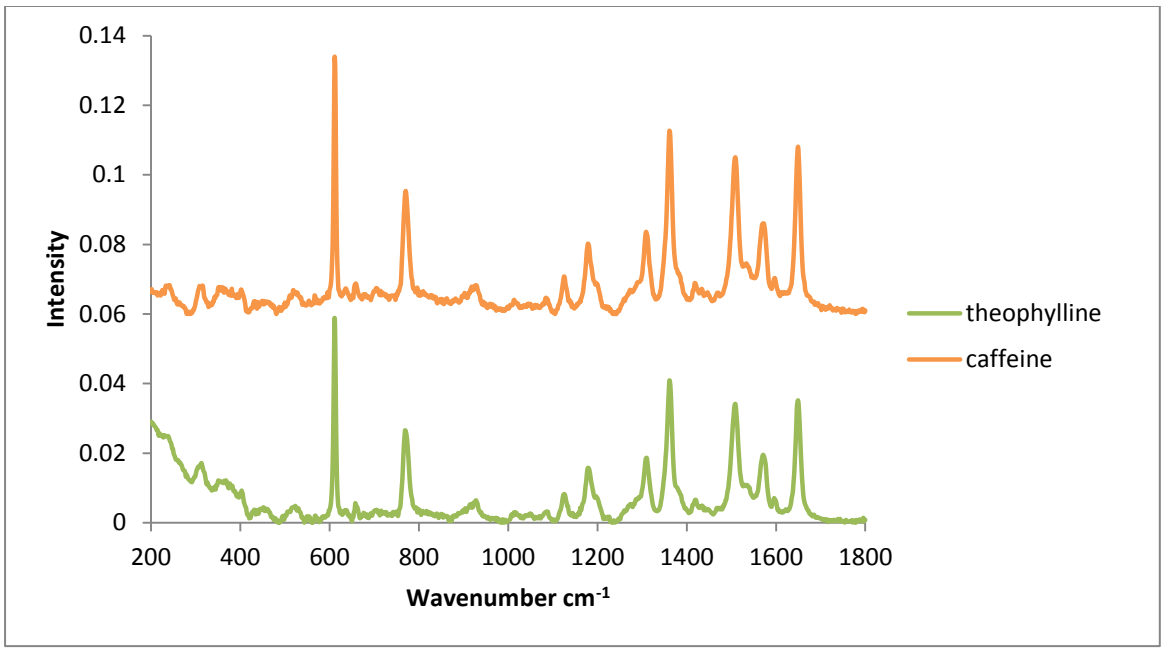


Figure 13: SERS spectrum of 25 µg/mL caffeine and theophylline with silver colloid and NaCl (0.1 M) Baseline corrected and normalised

If the spectra are compared from 400-1800 cm^{-1} the correlation coefficient of caffeine and theophylline has a value of 0.99, showing that the spectra of caffeine and theophylline are almost identical. This is due to the position of the molecule on the silver colloid surface. As previously explained the N-CH_3 group has the strongest interaction with the silver surface. This functional group is present in both caffeine and theophylline and therefore the SERS response is very similar between the two molecules as they are both behaving in the same way which explains why the spectra are similar.

4.7 Limit of detection (LOD) of caffeine in methanol using SERS

The limit of detection is calculated as it allows the lowest quantity of a substance to be distinguished from the absence of that substance ie a blank. To do this a calibration graph needs to be produced. One method of calculating the limit of detection is by looking at the mean value of a blank and the standard deviation of a blank. Table 14 shows the methanol blanks and the different peak heights.

Table 14: 10 replicates of MeOH blank and their peak heights at 1590 cm^{-1}, 1619 cm^{-1} and 1630 cm^{-1}

Replicates of MeOH blank	Baseline peak (BPB) (1590 cm^{-1})	Peak Height (1619 cm^{-1})	Baseline peak (BPA) (1630 cm^{-1})	PH – $\frac{(BPB + BPA)}{2}$
1	5.50	8.33	8.15	1.51
2	4.89	6.26	3.01	2.31
3	8.11	15.98	10.31	6.77
4	2.84	6.24	8.00	0.82
5	3.02	6.06	8.38	0.36
6	6.45	6.53	8.56	-0.98
7	5.22	8.87	5.61	3.46
8	2.20	6.44	6.38	2.15
9	2.82	3.21	1.61	0.99
10	2.04	5.86	4.42	2.63
			Mean	**2.00**
			SD	**2.10**

The LOD can be deduced using this equation:

- LOD = Mean $_{blank}$ + $3\sigma_{blank}$
- LOD = 2.00 + 3(2.10)
- LOD = 22.97 Raman intensity units

Once this has been established this would be substituted in as the y value and solve it for x which would then give the LOD in concentration. This would have been done however the calibration curve (figure 15) is not appropriate to use as it does not follow the linear trend expected (the response increases as the concentration increases) and the error bars overlap which means that being able to differentiate between the different concentrations is not

possible. Therefore the conversion from intensity units to concentration would not be accurate. Another method for determining the LOD is the use of standards.

Figure 14 shows the different concentrations of caffeine ranging from 10 ng/mL to 1 ng/mL. It is evident that as the concentration is decreasing so are the different bands. At 10 ng/mL there are some peaks that can be used to distinguish caffeine. However at 1 ng/mL the only peaks that are evident are the methanol peaks which can be found at 1000 cm^{-1} and 1450 cm^{-1}

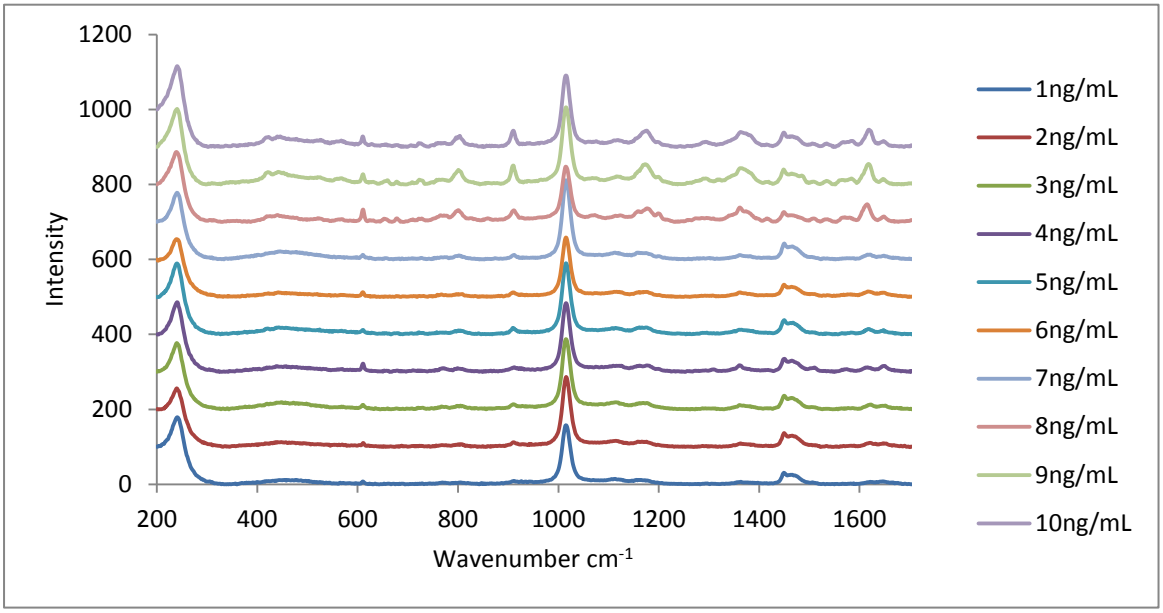


Figure 14: SERS spectra of caffeine at concentrations ranging from 10 ng/mL - 1 ng/mL

Table 15: The mean height of caffeine peak at 1619 cm^{-1} over a range of different concentrations

Concentration (ng/mL)	Mean *(n=10)*	Standard Deviation
10	30.79	12.33
9	55.56	7.61
8	45.37	13.99
7	23.25	6.43
6	15.63	1.39
5	18.53	3.85
4	14.41	0.97
3	15.79	2.14
2	16.70	1.75
1	16.04	4.28

If the sample is not above the blank + 3SD (22.97) then caffeine cannot be detected as it could be a blank measurement that is being seen. By looking at table 15, 10 ng/mL – 7 ng/mL are all above the limit of detection value. 6 ng/mL – 1 ng/mL are not. Therefore the limit of detection for caffeine can be concluded as being between 10-7 ng/mL. This conclusion enables the optimisation of the settings attempting to obtain a spectrum of these samples that contains the bands of caffeine and thus determining the LOD of caffeine in methanol.

Looking at table 16 it is clear that run 4 yields the highest caffeine to colloid peak ratio suggesting that it provides the best method that optimises the caffeine spectrum. This then enables the acquisition time to be looked at. To see if changing the amount of time the laser is exciting the sample has an effect on the spectrum produced. Table 17 shows the effect of changing the acquisition time for the analysis of caffeine. Run 1 evidently produces the best result as it yields the highest caffeine to colloid peak height ratio. These setting will now be used to determine the limit of detection for caffeine in methanol.

Table 16: The effect on the peak height ratio when changing the filter percentage and the quantity of sample added using 10 ng/mL of caffeine solution

Run	Filter (%)	Quantity of sample (*µl*)	Acquisition Time (seconds)	Colloid peak height at 200 cm^{1} (n=3)	Caffeine peak height at 1619 cm^{-1} (n=3)	Caffeine: Colloid peak height ratio (n=3)
1	25	100	30	44.35	67.54	1.52
2	25	200	30	80.51	84.18	1.05
3	25	300	30	52.04	84.58	1.63
4	**25**	**400**	**30**	**54.02**	**109.09**	**2.02**
5	50	100	30	53.03	38.43	0.72
6	50	200	30	93.02	34.82	0.37
7	50	300	30	86.39	36.49	0.42
8	50	400	30	42.20	6.01	0.14
9	100	100	30	50.01	39.73	0.79
10	100	200	30	114.24	69.40	0.61
11	100	300	30	262.69	171.34	0.65
12	100	400	30	1696.90	392.46	0.23

Using the parameters that have been highlighted in bold (run 4), the acquisition time can now be optimised

Table 17: The effect on the peak height ratio when changing the acquisition time

Run	Filter (%)	Quantity of sample (*µl*)	Acquisition Time (seconds)	Colloid peak height (n=3)	Caffeine peak height (n=3)	Caffeine: Colloid peak height ratio (n=3)
1	**25**	**400**	**30**	**54.02**	**109.09**	**2.02**
2	25	400	40	145.80	178.55	1.22
3	25	400	50	108.04	100.23	0.92

Once these settings have been optimised, they can be applied to caffeine to determine the limit of detection. Figure 15 shows the calibration chart of different concentrations of caffeine. It is clear from this figure that the limit of detection of caffeine is 8 ng/mL however due to the error bars overlapping considerably differentiating between 8 ng/mL, 9 ng/mL and 10 ng/mL cannot be done as the quantification limit will be a lot higher than the limit of detection, it normally is the mean $_{blank} + 10\sigma_{blank}$.

Looking at both methods for determining the LOD, they give similar results. The calculated LOD using intensity units was said to be 22.97, which is supported by figure 15. The concentrations that are below 7 ng/mL also have a Raman intensity below 22.97.

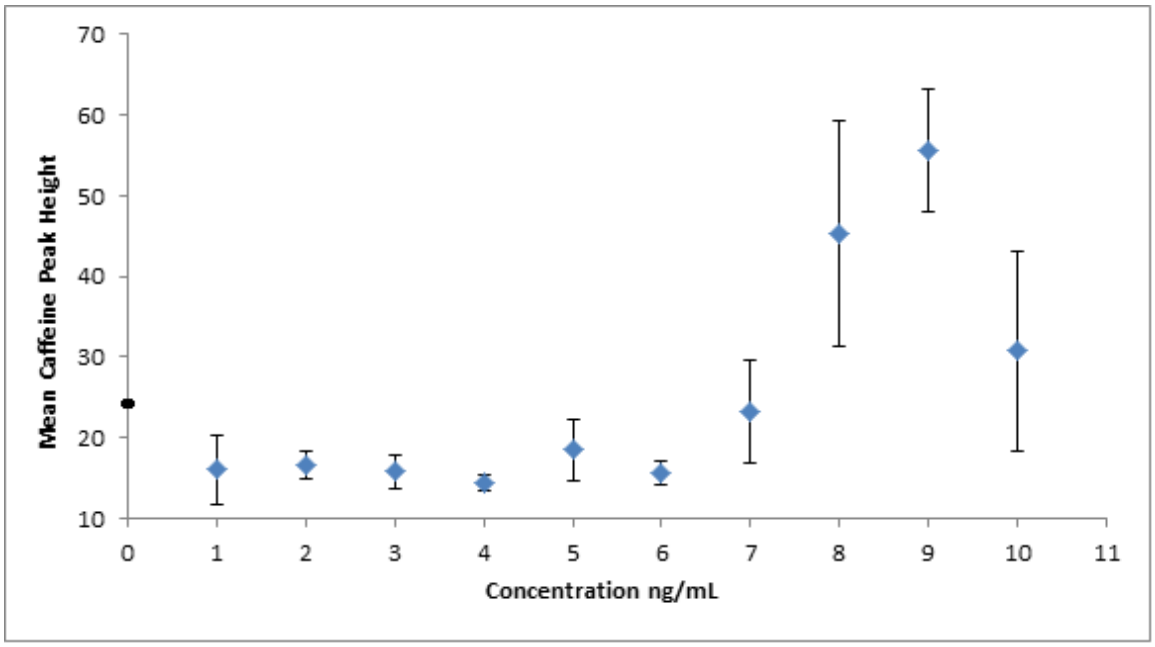


Figure 15: Calibration chart looking at different concentrations of caffeine in methanol. The black dot represents the peak height of the blank methanol (n=3, error bars are ±1SD)

Higher concentrations of caffeine were looked at to see if it is possible to determine the quantity present. Looking at figure 16 it is clear that there is too much variation between the different concentrations. This could be due to a repeatability issue with the colloid and its batch-to-batch variability which could result in a different amount of silver chloride being added, different number of hot spots and different sizes of aggregates. One colloid can produce 10 samples that can be analysed. During this experiment a minimum of 3 batches was used. In future it would be ideal to make them individually, analyse them on the UV-Vis to determine their suitability and then pool them together to create a colloid that is stable.

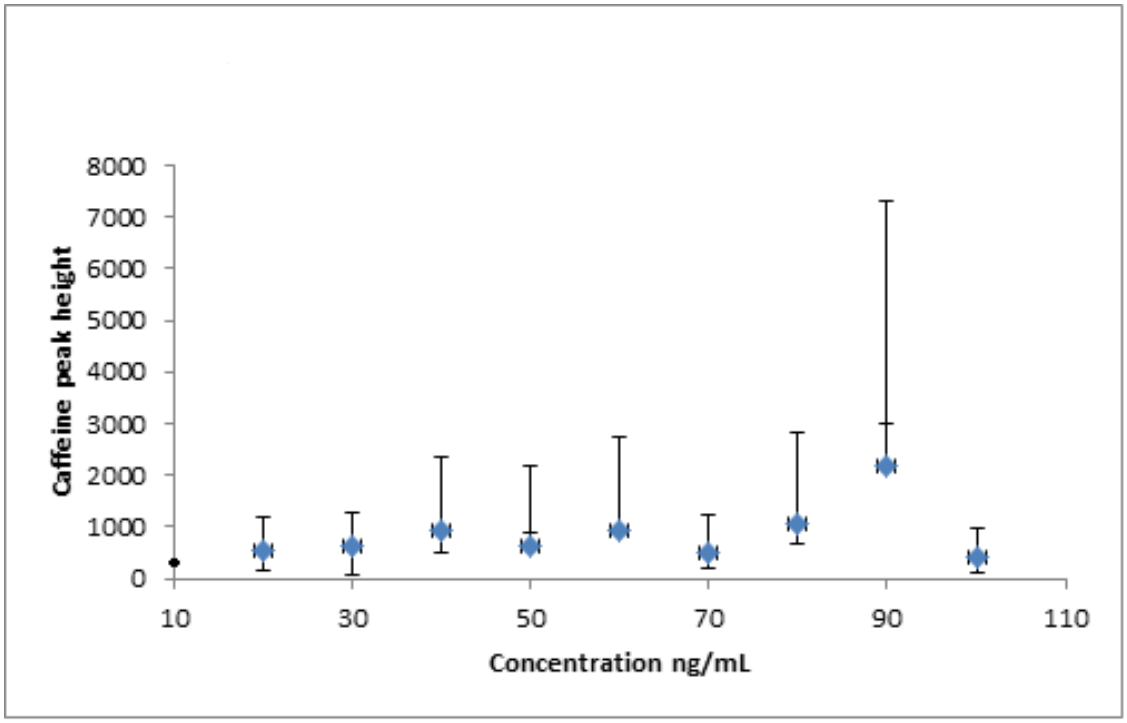


Figure 16: Calibration chart looking at different concentrations of caffeine in water ranging from 20 ng/mL - 100 ng/mL. The black dot represents the peak height of the blank methanol (n=3, error bars are ±1SD)

4.8 Optimising High-Performance Liquid Chromatography HPLC for the analysis of caffeine in methanol

To optimise the HPLC method, caffeine and theophylline were analysed to determine if the different parameters provided good separation of the two molecules. The different parameters that were looked at were the retention time, peak height, peak area and peak shape. The retention time was looked at to see if it varied due to the different parameters. Peak area was analysed because it is consistent within the HPLC and relates to concentration. The peak height is measured to enable the peak shape to be calculated. The peak shape refers to the symmetry of the peaks on the chromatogram and whether there was any peak fronting or tailing.

The flow rate was changed to see if there was a change in the retention time of the two substances and to see whether better separation could be achieved. Table 18 summarises the changes in parameters.

Table 18: Different parameter changes and their effect on the retention time (RT), peak height, peak area and peak shape

Change in Parameter		RT (min)		Peak height		Peak Area		Peak shape (area/height)	
		Theophylline	Caffeine	Theophylline	Caffeine	Theophylline	Caffeine	Theophylline	Caffeine
Flow rate (mL/min)	1.5	2.03	2.27	205544	1587663	9582950	12426232	46.62	7.83
	1.0	2.69	3.01	1447364	3027411	13114597	16441257	9.06	5.43
	0.5	5.31	5.95	1611438	3424491	24208632	31309265	15.02	9.14
	0.1	23.97	26.92	2417713	3293635	130974875	159049782	54.15	48.29
Injection volume (µl)	5	2.77	3.09	1360942	1423800	8637718	11784508	6.35	8.28
	10	2.69	3.01	1447364	3027411	13114597	16441257	9.06	5.43
	15	2.69	3.07	1813052	3857884	16250333	21812983	8.96	5.65
	20	2.69	3.07	2334641	4661989	20307367	28145480	8.70	6.04
oncentration (Theo:Caff µg/mL)	25:100	2.69	2.99	552564	2775530	2593307	16177745	29.28	5.83
	50:75	2.69	2.99	1089853	1906802	4892276	9308480	8.54	4.88
	75:50	2.69	2.99	1704555	1326938	8012427	7057271	4.14	5.32
	100:25	2.69	2.99	2293554	670880	11554061	4650312	2.03	6.93
Mobile phase MeOH:water)	100:0	2.69	3.01	1447364	3027411	13114597	16441257	9.06	5.4
	90:10	2.72	3.04	1748924	3056078	13071244	16295773	7.47	5.33
	80:20	3.33	3.71	1096044	1350760	6171842	7977296	5.63	5.91
	70:30	3.65	4.16	801191	751353	6462166	6894351	8.07	9.18

The flow rate at 0.1 mL/min provides the best peak shape. The chromatogram also showed baseline separation and the peaks were sharp and clean. When comparing the different retention times, they have increased as the flow has decreased which is what was expected. It also shows that there is a longer separation between when theophylline and caffeine elute. Using these results, the 0.1 mL/min flow rate is the best flow rate to use when analysing these two substances.

When analysing the different injection volumes, the 20 µl volume provided the best separation with the cleanest peaks. The other three injection volumes did not provide baseline separation and the peaks were not symmetrical. The results for the different volumes also show that the peak area increases with the amount that is injected again something that was expected.

The concentrations were changed to see the effect on the peak area. Table 18 shows that when the concentration is increased of one of the substances the peak area increases. It also shows that changing the concentrations does not have an effect on the retention time. The reason concentrations were looked at is because when analysing a sample the concentrations are not necessarily going to be the same for the substances and its metabolites.

The mobile phase was looked at as it can influence the spectrum which is shown in the table. When changing the mobile phase it changes the polarity so the substances should elute at different retention times which is visible in the results table. Another thing that changed was the baseline separation, there was complete separation with clean, sharp peaks with the 70:30 (MeOH:H_2O) mobile phase as oppose to the 90:10.

To summarise the best method to use for the analysis of caffeine is a flow rate of 0.1 mL/min, a mobile phase of 70:30 and an injection volume of 20 µl as this gave the best separation of caffeine and theophylline.

4.9 Limit of Detection of Caffeine using HPLC

Using the optimised HPLC method the limit of detection of caffeine was determined. Figure 17 shows chromatograms of different concentrations of caffeine. A peak at 2.7 minutes can be seen which is due to caffeine. As the concentration of caffeine decreases, the peak height of caffeine decreases. Looking at the chromatogram of caffeine at a concentration of 0.1 µg/mL it is clear that the peak at 2.7 min is no longer distinguishable. This suggests that the limit of detection of caffeine using these HPLC settings is 0.2 µg/mL.

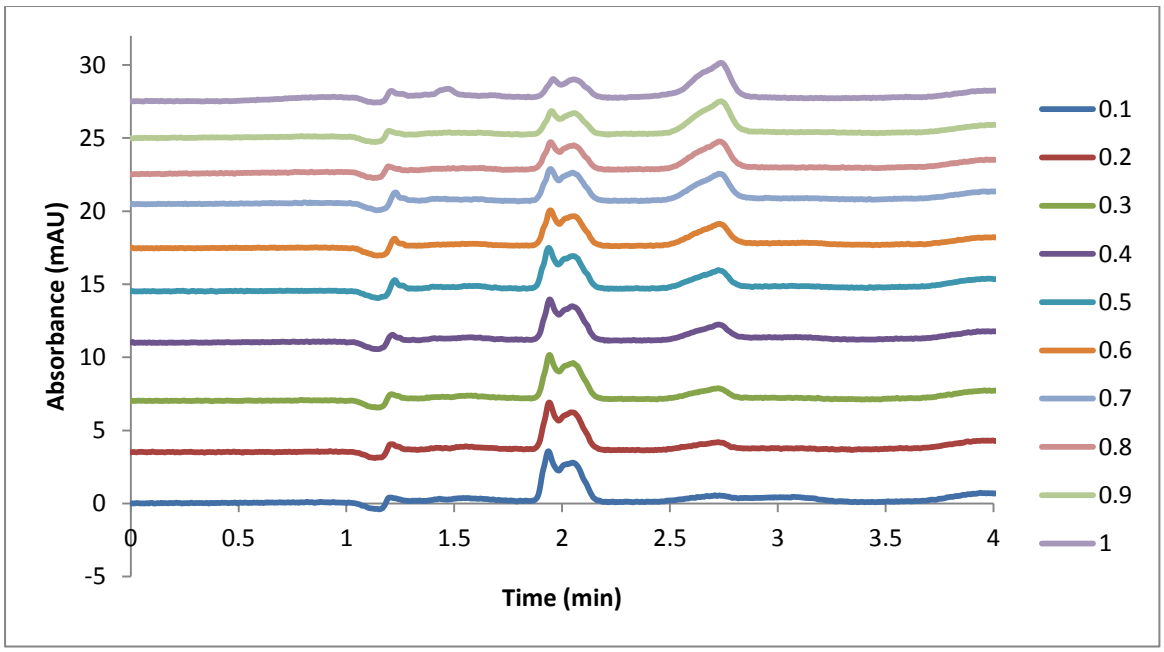


Figure 17: Concentrations of caffeine in methanol ranging from 0.1 µg/mL - 1 µg/mL using the optimised HPLC settings.

Figure 18 shows a calibration curve that was produced by plotting the peak area against known concentrations of caffeine. Table 18 shows the results used to produce the calibration curve.

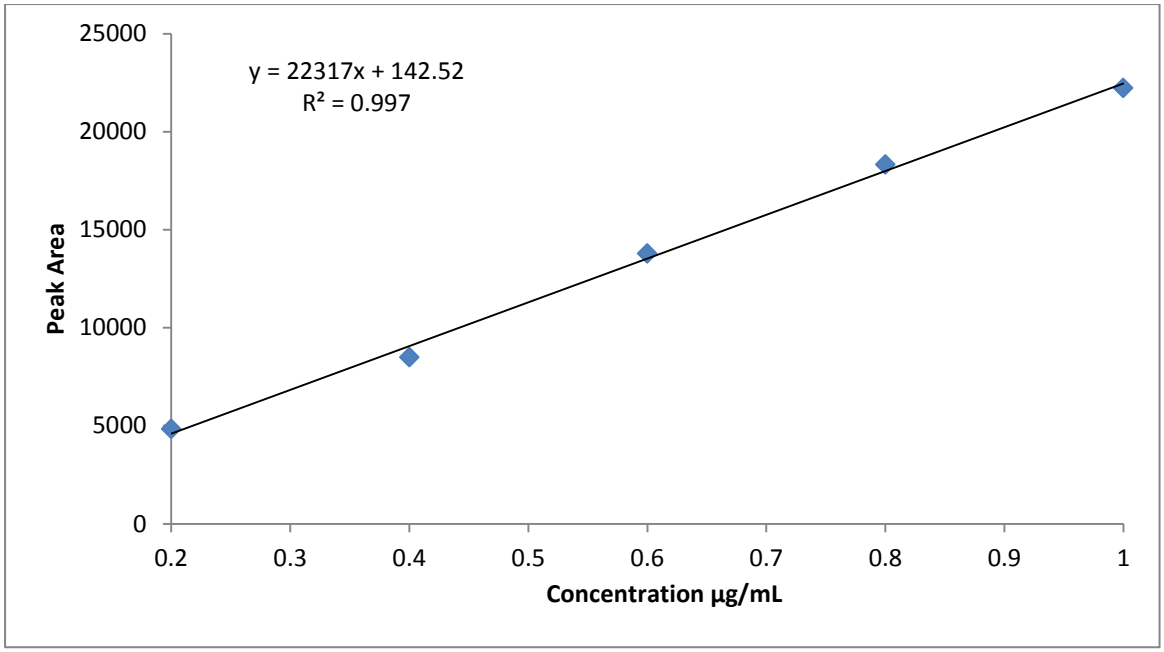


Figure 18: Calibration curve showing different concentrations of caffeine in methanol (n=3, error bars are ±1SD and are within the size of the point)

Table 19: Peak area of caffeine in methanol at different concentration with 3 replicates

Concentration (µg/mL)	Rep 1	Rep 2	Rep 3	Mean	Standard Deviation
1	22248.98	22239.43	22203.45	22230.62	24.01
0.8	18337.54	18324.15	18322.27	18327.99	8.33
0.6	13596.75	13930.90	13813.79	13780.48	169.56
0.4	8425.84	8460.65	8589.98	8492.16	86.49
0.2	4894.23	4801.89	4799.12	4831.75	54.13

Figure 19 shows a R^2 value of 0.997 which shows that there is a linear relationship between the peak area and concentration. The R^2 value depicts the quality of the gradient of the line. For quantitative techniques the desired R^2 value is 0.98 or above. Other quantitative methods look at the use of an internal standard. An IS can be used to help build a calibration, by plotting the ratio of the signal from the analyte of choice to the signal from the IS which can aid the quantification. Factors that affect the signal of the analyte will affect the signal of the IS so the use of an IS can help correct for any loss of analyte during the sample preparation or sample injection. An IS normally has a similar chemical structure to the analyte. When analysing caffeine there are a number of different analytes that can be

used as an internal standard. A common IS used is theophylline due to it having a very similar structure to caffeine, however as theophylline is a metabolite of caffeine it could be present in the sample matrix. Therefore theophylline is not a suitable internal standard for the analysis of caffeine in saliva. Another analyte that has been used in the literature is benzotriazole, which was used for the analysis of caffeine and paraxanthine in saliva and plasma on a HPLC (Perera et al., 2010). When using this internal standard along with the optimised HPLC settings both caffeine and benzotriazole co-elute, therefore making it unsuitable to use for this analysis. Another IS that could be used is paracetamol.

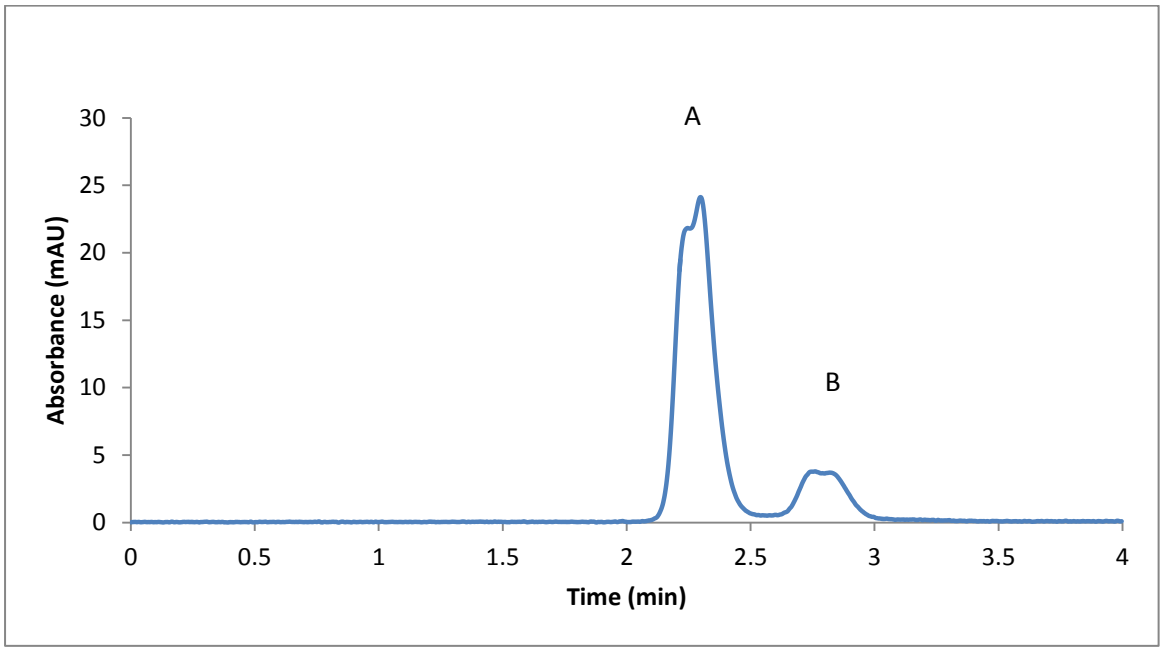


Figure 19: Chromatogram of paracetamol (A) and caffeine (B)

Looking at figure 19 it is clear that both paracetamol and caffeine have complete baseline separation and different retention times. Therefore paracetamol can be used as a suitable internal standard, however in this case it is not. There is a possibility that paracetamol could be present in saliva, especially if the participant providing the sample has had paracetamol before the sample was taken. Another internal standard that could be used are the analogues of paracetamol; 2-acetamidophenol and 3-acetamidophenol. Figure 13 shows the chromatogram of caffeine and 3-acetamidophenol. By comparing the two different chromatograms there is not complete baseline separation, this could be improved by modifying the method (see section 6.1.1). However 3-acetamidophenol does have a similar

retention time to that of paracetamol again this would create a problem if the participant has taken paracetamol prior to providing a saliva sample.

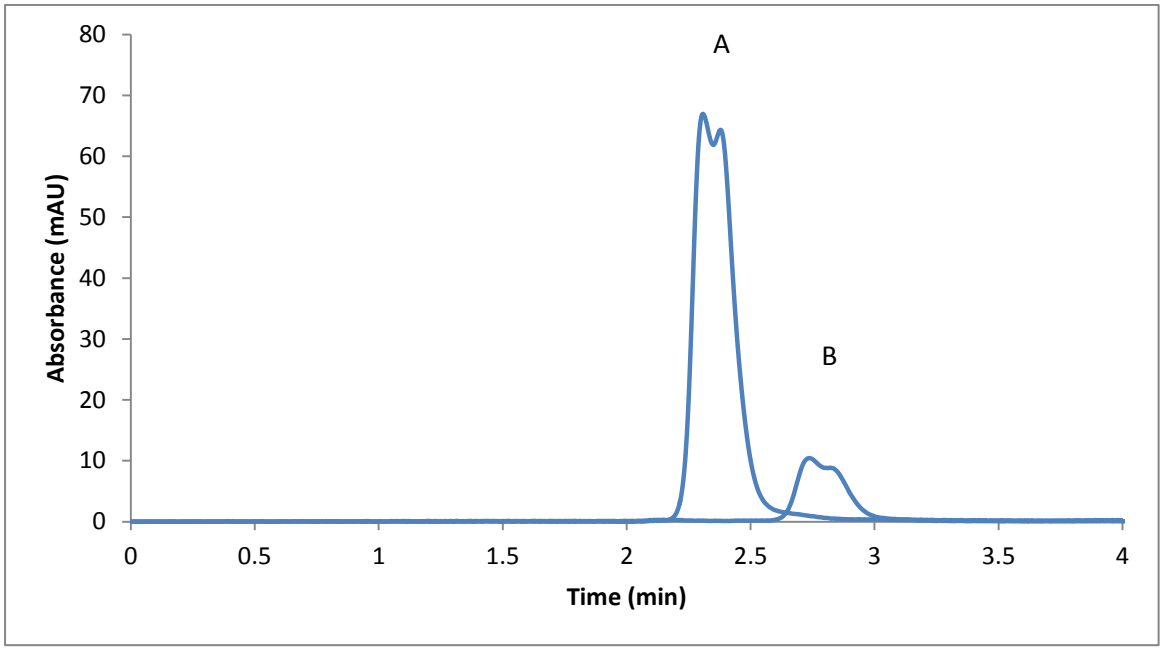


Figure 20: Chromatogram of 3-acetamidophenol (A) and caffeine (B)

2-acetamidophenol was also considered as an internal standard (figure 21). Using the optimised method there is no baseline separation between 2-acetamidophenol and caffeine therefore making it unsuitable.

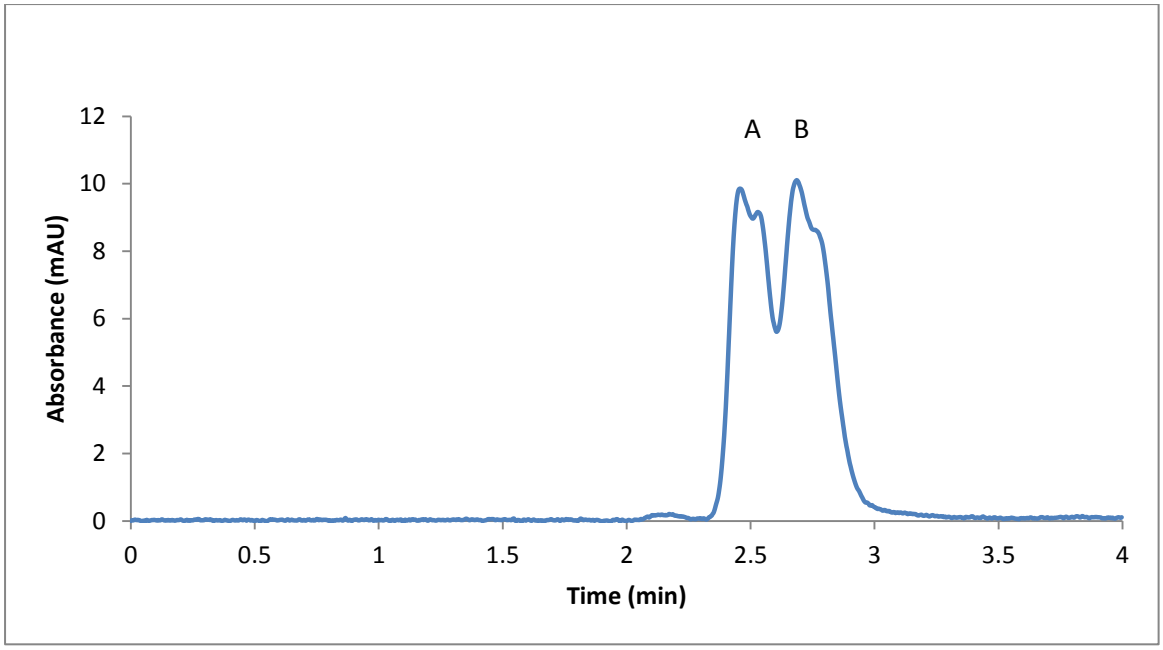


Figure 21: Chromatogram of 2-acetamidophenol (A) and caffeine (B)

The use of an internal standard for this piece of work would be unsuitable as the internal standards that have been investigated either co-elute or are not baseline separate. To improve the baseline separation modification of the existing HPLC method would need to be undertaken (section 6.1.1). When looking figure 18 it shows there is a good linearity between the peak area of caffeine and the concentration because of this the use of an internal standard wasn't investigated further as the calibration curve is suitable for quantitative analysis.

4.10 Liquid-Liquid Extraction (LLE) Technique

A LLE technique for the determination of caffeine in non-alcoholic beverages was adapted for the analysis of caffeine in water (University of Lincoln, 2005). Caffeine in water was used as a means to test whether the extraction was suitable for the extraction of caffeine in saliva. To determine whether the extraction would be suitable a blank was analysed. As can be seen by Figure 22 there is some contamination (1-1.5 minutes) which is caused by the extraction technique. However there are no peaks at 2.5 minutes which is the time at which caffeine elutes. It can be concluded that the LLE is capable of analysing caffeine without any interfering peaks.

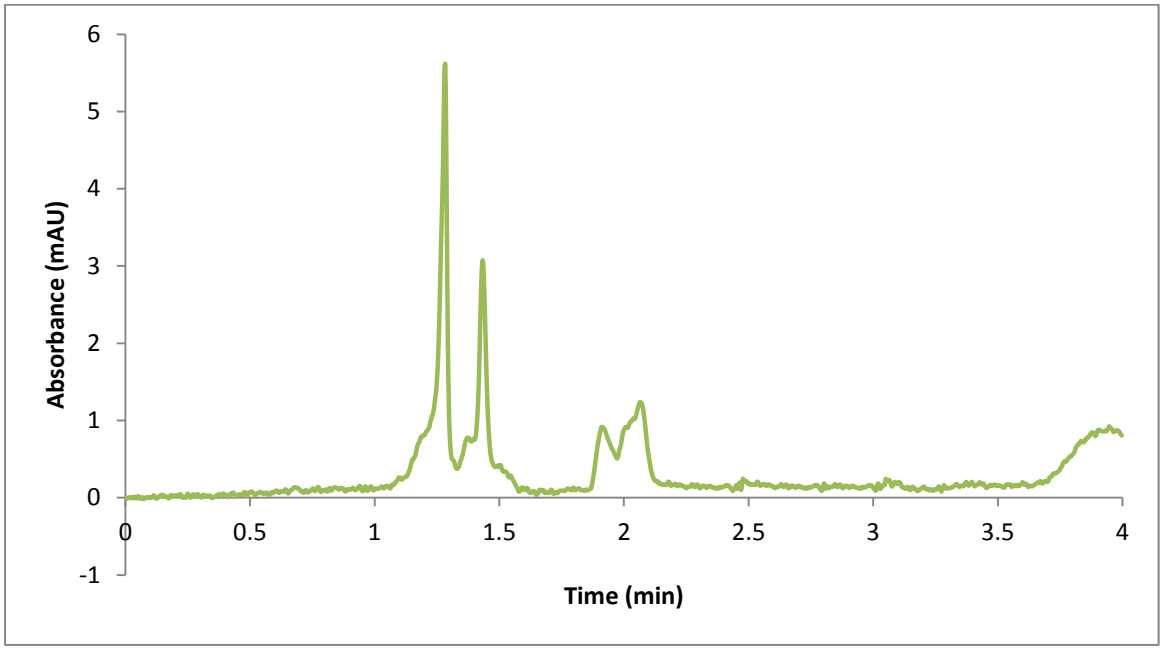


Figure 22: HPLC chromatogram of a blank sample (water) analysed after Liquid-Liquid Extraction

Figure 23 shows the chromatogram of the LLE with a known concentration of caffeine. This demonstrates that the extraction technique is capable of extraction of caffeine in water. There are peaks present at 1 minute which can be concluded as something that has transferred over from the extraction technique. This can be deduced as the same peaks are present in the blank chromatogram (Figure 22) as well as the 1 µg/mL chromatogram below (Figure 23).

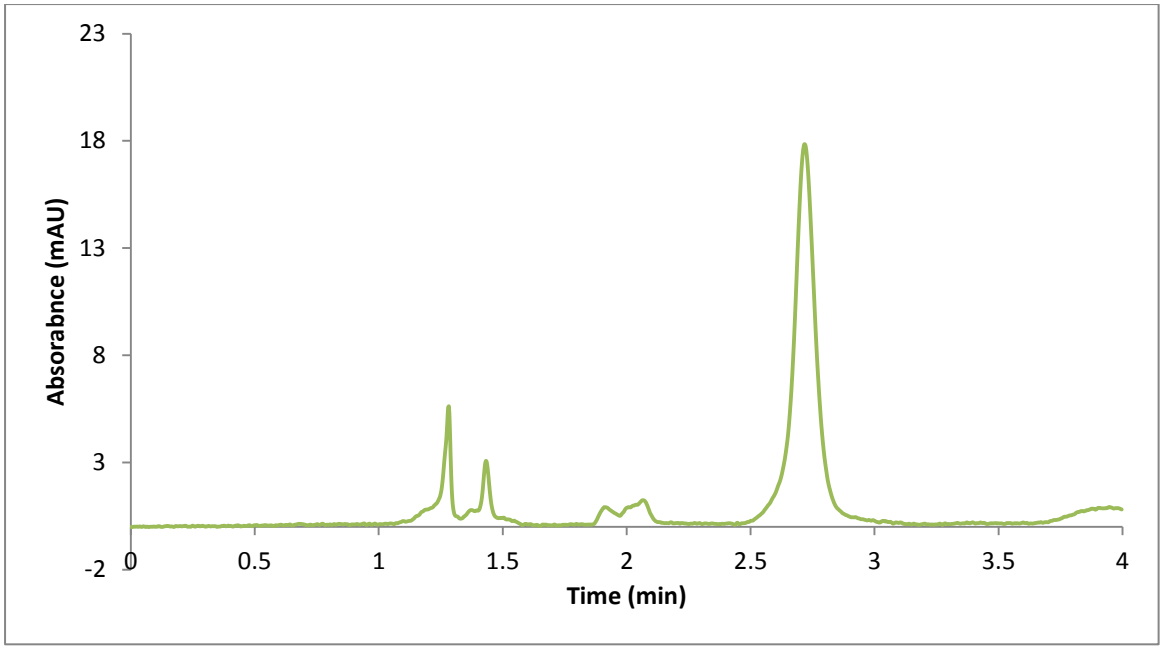


Figure 23: HPLC chromatogram of 1 µg/mL of caffeine in water analysed after Liquid-Liquid Extraction

Different number of extractions was looked at to optimise the extraction technique. It can be concluded that two extractions is enough to fully extract the caffeine from the sample (Figure 24).

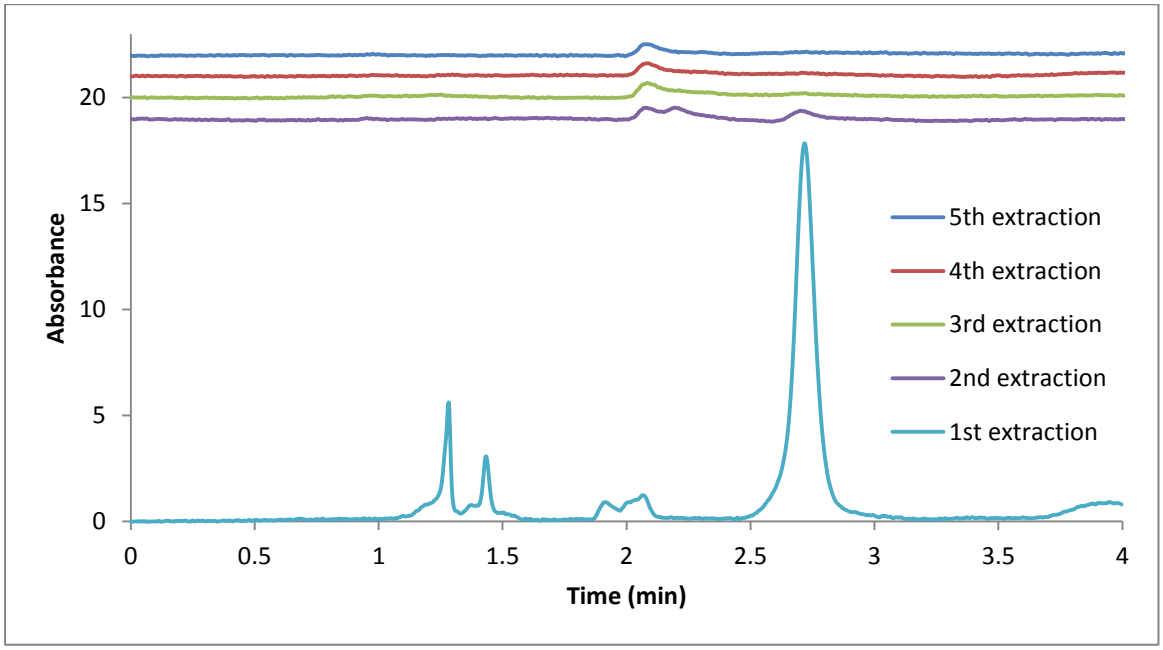


Figure 24: HPLC chromatogram of different number of extractions using a 1 µg/mL sample of caffeine in water

As can be seen by figure 24 there are no peaks visible in the 3rd, 4th and 5th extraction apart from the methanol peak at 2 minutes, which would be expected, therefore concluding that 2 extractions is enough to successfully extract caffeine. Once the extraction method has been optimised a calibration curve was produced for caffeine in water (Figure 25).

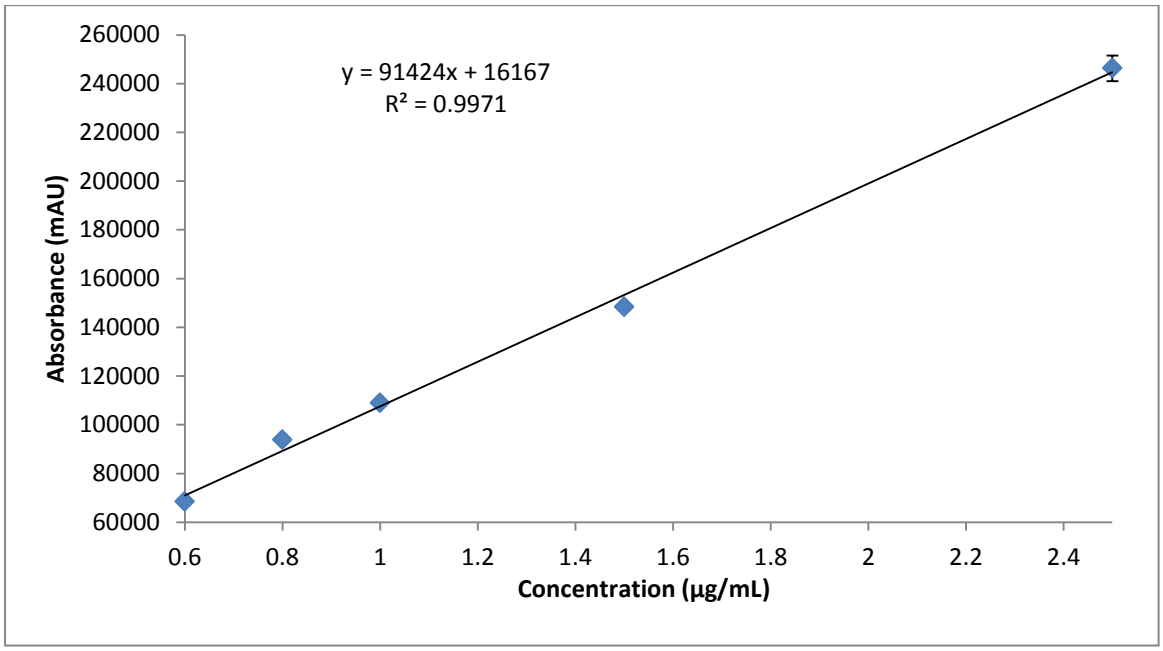


Figure 25: Calibration curve of a liquid-liquid extraction of different concentrations of caffeine in water ranging from 0.6 - 2.5 µg/mL (n=3, error bars are ±1SD and are within the size of the point)

4.11 Analysis of Caffeine in Saliva

To be able to quantify caffeine in saliva a calibration curve needs to be produced that can be used to help quantify participants' saliva samples. Caffeine free saliva was collected from myself throughout the experiment. No caffeine was consumed for over 24 hours before the saliva sample was taken and so this was assumed to be caffeine-free. Figure 26 shows the calibration curve of caffeine in saliva.

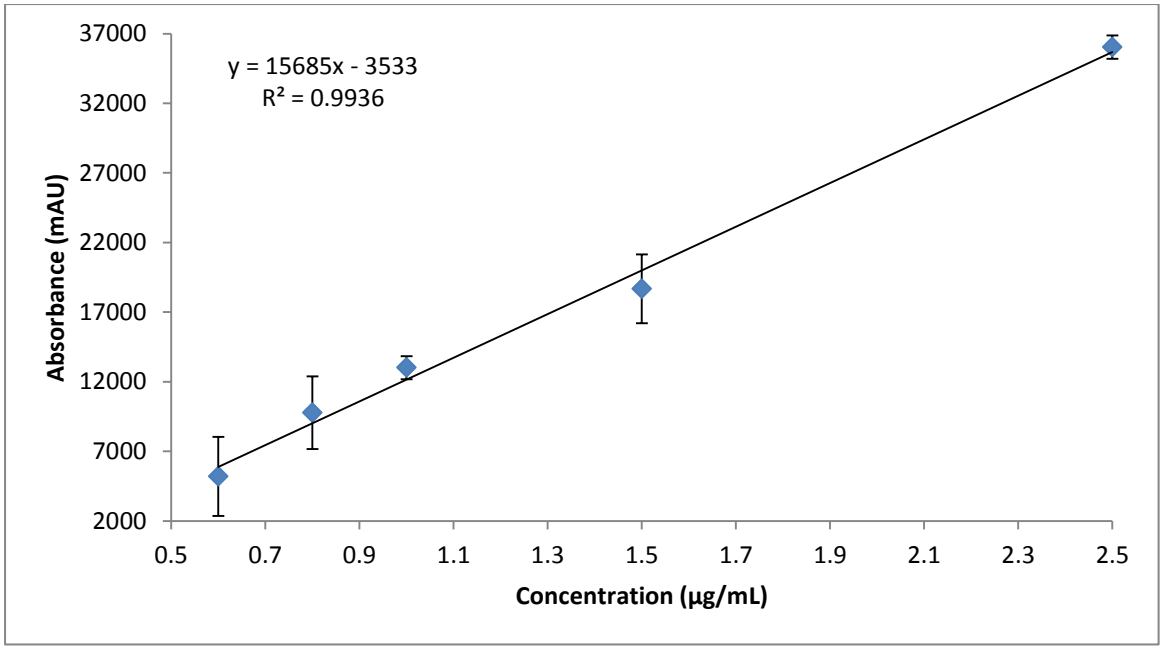


Figure 26: Calibration curve of a liquid-liquid extraction of different concentrations of caffeine in saliva ranging from 0.6 - 2.5 µg/mL (n=3, error bars are ±1SD)

Validation was under taken to determine the precision and the accuracy of the calibration curve that will be used for the quantification of caffeine. Three independent samples (top, middle and bottom) at known concentrations were prepared and analysed on the HPLC. They were injected in duplicate and an average was taken which was then used to predict the amount of caffeine present.

Table 20: Calculations for the validation set ranging over 2 days

Sample of known concentration (µg/mL)	Day 1			Day 2	
	Average peak area	Standard Deviation	Concentration (µg/mL)	Average peak area	Concentration (µg/mL)
1.0	12553.91	315.10	1.02	-	-
1.7	24430.32	464.70	1.78	-	-
2.5	35015.28	120.14	2.46	-	-

Table 20 demonstrates that day one of the validation there is a slight bias towards a high results. When the samples were analysed on day two, there was no caffeine present in any of the vials suggesting that there could have been an instrument failure ie miss-injection. It

is unlikely that caffeine degraded over night because Perera et al (2010) concluded that caffeine was stable refrigerated at 4°C and room temperature for 14 days. This put an end to the validation set. Ideally the validation should be done over a number of days demonstrating the precision of the calibration and the accuracy.

To determine if the two different matrices are comparable the calibration graph of caffeine in water was plotted against the calibration graph of caffeine in saliva (Figure 27).

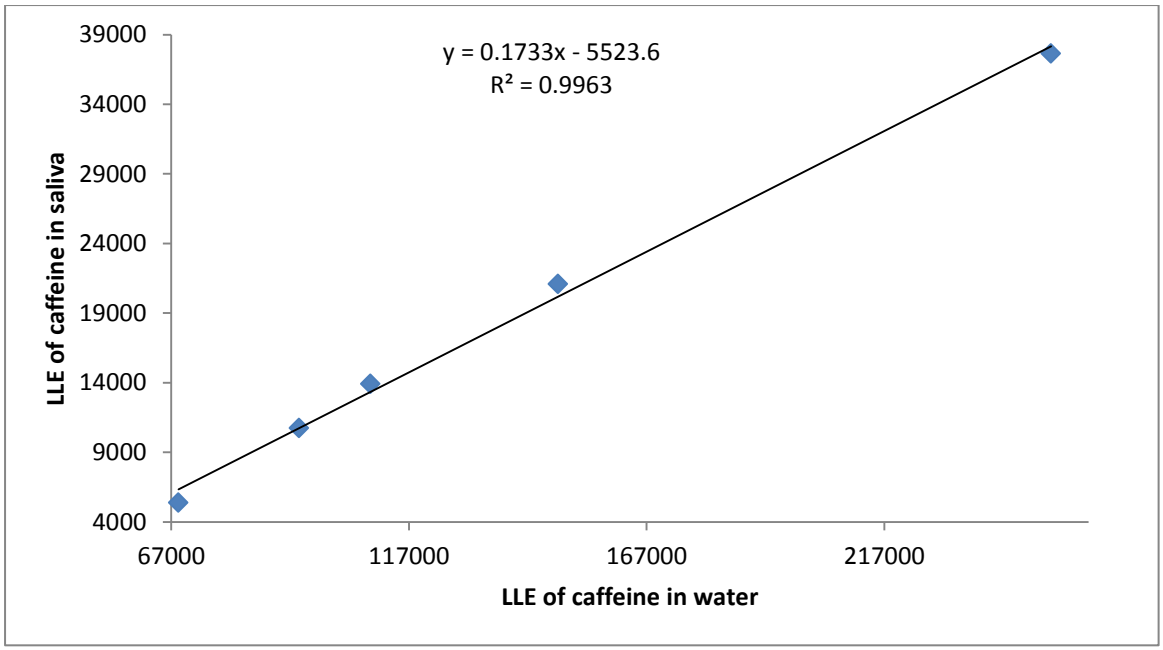


Figure 27: Calibration curve comparing the peak areas of caffeine in water against the peak areas of caffeine in saliva (n=3, error bars are ±1SD and are within the size of the point)

The R^2 value is 0.9963 which shows that for the same concentration the signal increases in the same way for both techniques. If there was a gradient of 1 it would show that the two techniques are the same, the same response is obtained from saliva and water. However the signal is much more reduced in saliva suggesting that there is a matrix effect. This suggests that the caffeine is not as easily extracted from saliva as it is in water which could be down to a number of reasons, for example the proteins that are found in saliva could be having an effect on the caffeine extraction. The equation of the line seen in Figure 26 was the equation used to quantify the caffeine in saliva.

4.12 Analysing Saliva samples from participants

A consent form was given to the participants explaining what the experiment included and assured them that no medical information would be gathered from this experiment. Each participant was given a unique code to keep their identity anonymous (see section 8). Before analysing the saliva samples from the participants, they were asked to go 24 hours without any form of caffeine to ensure that the result that is gathered is down to the caffeine that was consumed when the saliva was given. To ensure that each participant had no caffeine in their system before the start of the experiment a saliva sample (blank) was taken, they then consumed a normal cup of coffee and after half an hour their saliva was collected in the salivettes. An average cup of coffee contains approximately 85 mg of caffeine (McCusker et al., 2003). It was then extracted, blown down to dryness and reconstituted in methanol. The sample was then analysed by SERS and by HPLC. However when the samples were analysed there was no caffeine response. Ideally the saliva could have been analysed directly to the colloid. The direct analysis of caffeine in saliva was not looked at because when trying to analyse caffeine in water it was unsuccessful so the assumption was that direct analysis would not be successful as saliva is 99% water. This is explained further in the further research section (section 6). The optimised HPLC method was used to quantify the caffeine content. Figure 28 and 29 shows an example of one of the participant's blank saliva sample and their saliva sample after the consumption of caffeine.

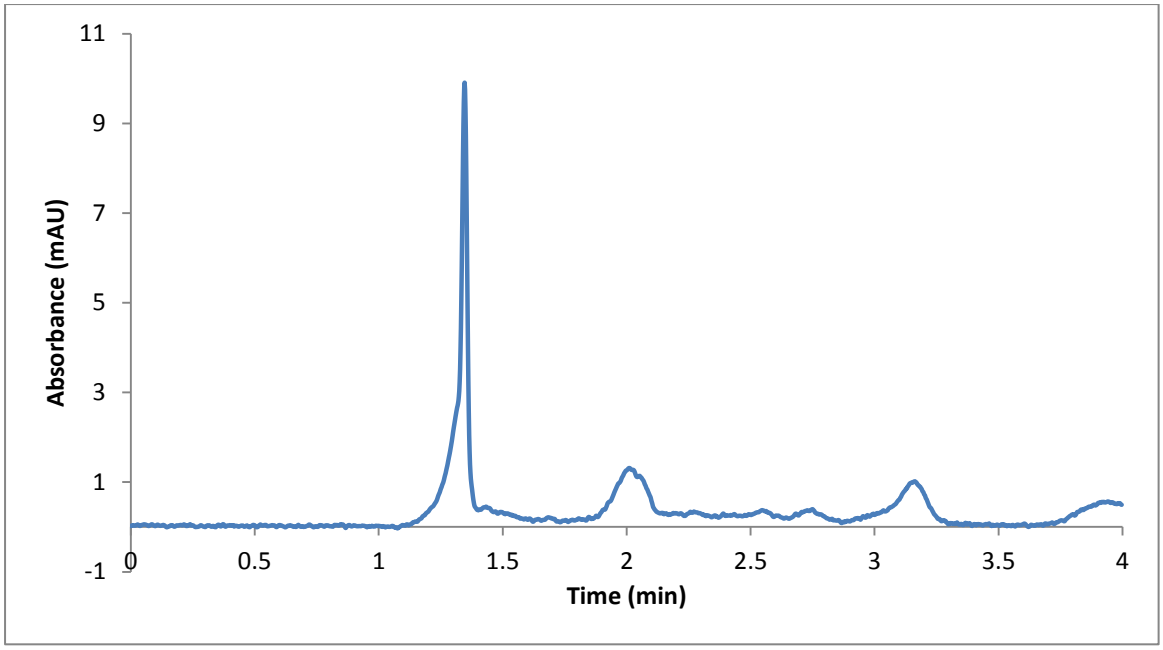


Figure 28: Participant A3B8's blank saliva sample

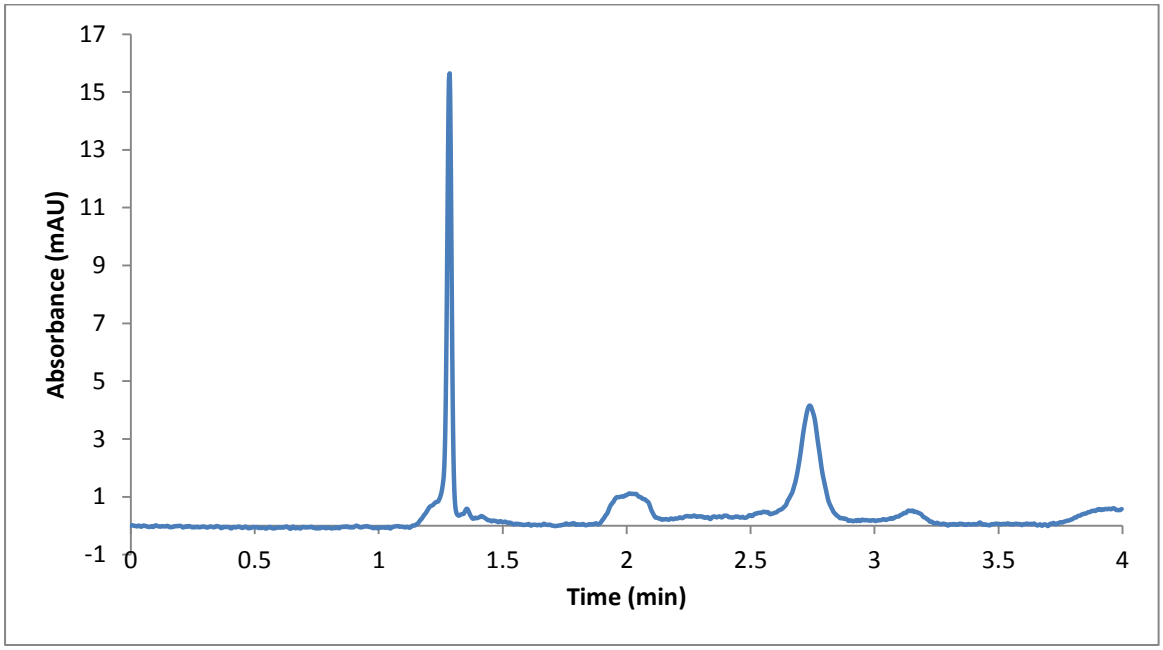


Figure 29: Participant A3B8's saliva sample after the consumption of caffeine

Comparing both figure 28 and 29 it is clear that there was no caffeine present in the blank saliva sample and that the presence of caffeine in figure 29 is down to the caffeine consumed that day and not from prior caffeine consumption. All of the samples were analysed on the HPLC in duplicate then an average was taken. The equation that is in figure

26 was rearranged in order to calculate the concentrations of the caffeine for each individual. There is an example of the equation being used in appendix 2 (see section 8).

Table 21: Calculation for the concentration of caffeine in saliva for 8 different participants (n=2)

Participant	Average peak area	Calculated concentration (µg/mL)
A3B8	24033.160	1.76
A5B5	26705.735	1.92
A6B3	24894.340	1.81
A9B2	21128.365	1.57
A1B1	26850.560	1.94
A5B3	20115.897	1.51
A2B9	23094.074	1.70
A7B1	25956.524	1.88

It can be concluded that the analytical HPLC method is capable of determining concentrations of caffeine in different individual's saliva. Chloroform is not a good extracting solvent for proteins and they are unlikely to be present in the caffeine extracts. It is more likely to be other chloroform soluble substances that are extracted that may be interfering with the SERS analysis. This can be confirmed when looking at figure 29; there are some interfering peaks that can be seen in the chromatogram which could be stopping the caffeine from binding to the hotspots. Table 21 shows that the range of caffeine in saliva is between 1.51-1.94 µg/mL, the limit of detection for the optimised SERS method was significantly lower than the concentrations that were found in saliva. This suggests that the quantification using SERS could be possible if the calibration standards were at a concentration that reflects the amount of caffeine found in saliva.

5. Conclusions

It can be concluded that this research has efficiently been able to confirm the presence of caffeine in low quantities. This highlights that Surface Enhanced Raman Spectroscopy is a more sensitive technique for looking at lower limits of detection of specific analytes compared to Raman spectroscopy. The original aim of this research was to develop a SERS method capable of quantifying caffeine in saliva. However this proved problematic for a number of reasons discussed in the further research section. SERS was then going to be used as a confirmation technique. However using SERS as a technique to confirm the presence of caffeine in saliva samples was unsuccessful. More investigations into the reasons why SERS did not work would need to be studied. This research was also able to produce a HPLC method for the analysis of caffeine in saliva and quantify that amount of caffeine in different individual's saliva.

6. Further research

6.1 The use of an Internal Standard

6.1.1 HPLC method optimisation

The HPLC method has proven its capability of being able to quantify caffeine in saliva. One thing that could have been done to improve the HPLC method is the use of an internal standard (IS). A number of different internal standards were looked at to use with this study, however they either co-eluted with caffeine or eluted too close together that there was no base line separation. There are a number of ways to improve this, an IS that would not co-elute would be the best however it would need to be a standard that does not naturally occur in the body and is not a bi-product of caffeine. Using theophylline would not be suitable as it is a bi-product of caffeine along with theobromine. Paracetamol however did provide baseline separation (see section 4.9) but as explained previously if any of the participants have taken paracetamol prior to the investigation it would affect the result obtained. One other way to improve the method would be to optimise the method so that baseline separation could be obtained between either 2-acetamidophenol or 3-acetamidophenol and caffeine. This could be done by looking at the flow rate and modifying it accordingly until baseline separation is obtained.

6.1.2 Liquid-liquid extraction

By using an internal standard this could have been added to the saliva sample before the caffeine had been extracted, by doing this it not only would have aided the quantification as a peak area ratio would have been taken rather than relying just on the peak area of caffeine it would have also meant that the percentage recovery could be monitored in the extraction. If a known concentration of internal standard is added and the same amount is extracted and quantified than it can be certain that 100% of the IS is being recovered and therefore 100% of the caffeine is also being recovered.

6.2 SERS method

6.2.1 Optimisation of the colloid

One of the problems that was observed during this research was the repeatability of the colloid. As Peter White explained there is a problem with producing colloid solutions that have a good batch-to-batch reproducibility and stability. This was observed when preparing the colloid solutions, if the steps were not followed in the precise way and they were not done accurately than the colloid does crash. It takes a great level of skill in order to be able to produce repeatable colloids, if the hydroxylamine phosphate was not added straight and in the middle of the vial the colloids had a tendency to come out of solution. One way to improve the repeatability of the colloid would be to pool all of the individual vials together that way the individual colloid solutions would be mixed together and made uniform.

6.2.2 Optimisation of caffeine in water

To improve the method used saliva could have been added directly to the colloid rather than having to use an extraction technique and reconstitute in methanol. However during this research when caffeine was analysed in water with SERS a spectrum was not obtained. An assumption was made that if caffeine in water would not work then caffeine saliva would not work either. In order to improve the SERS method it needs to be optimised with regards to caffeine in water. As the majority of saliva is water, water could have been used to optimise a method. The aggregation needs to be considered as it plays a key part in the enhancement; if caffeine was not “sticking” to the SERS hotspots then a spectrum would have not been obtained.

6.3 HPLC method

When trying to validate the HPLC method on day 1 the results obtained were close to the actual amount that was injected. However on day two there were no peaks present from the caffeine samples. One improvement would be to determine the shelf life of caffeine and determine what temperature is best to store caffeine.

6.4 Quantitative method using SERS

A common method that is being looked at to quantify concentrations using spectroscopy is multi-variant analysis (Khoshayand et al., 2008). One of the most frequent used methods for

multi-variant analysis is partial least squares regression (PLS) which is based on latent variable decomposition. There are two different procedures to use; PLS1 and PLS2. PLS1 is one model that is built for each analyte and PLS2 is a calibration model that is constructed of all the analytes of interest (Khoshayand et al., 2008). In order to build a model reference data would need to be collected. In this case, a set of caffeine standards would be selected and scanned using SERS then analysed on the HPLC to obtain the 'actual' concentration value. This would be done for both the standards and samples. Using the correct software the scanned samples would be plotted against the actual concentration obtained by the HPLC. This would then be the calibration model which can be optimised by looking at the use of different regions for example the spectral regions that are associated with caffeine would be selected, different principal components and different smoothing tools. Once the model is built and validated, it can be used to predict the concentration of unknown samples.

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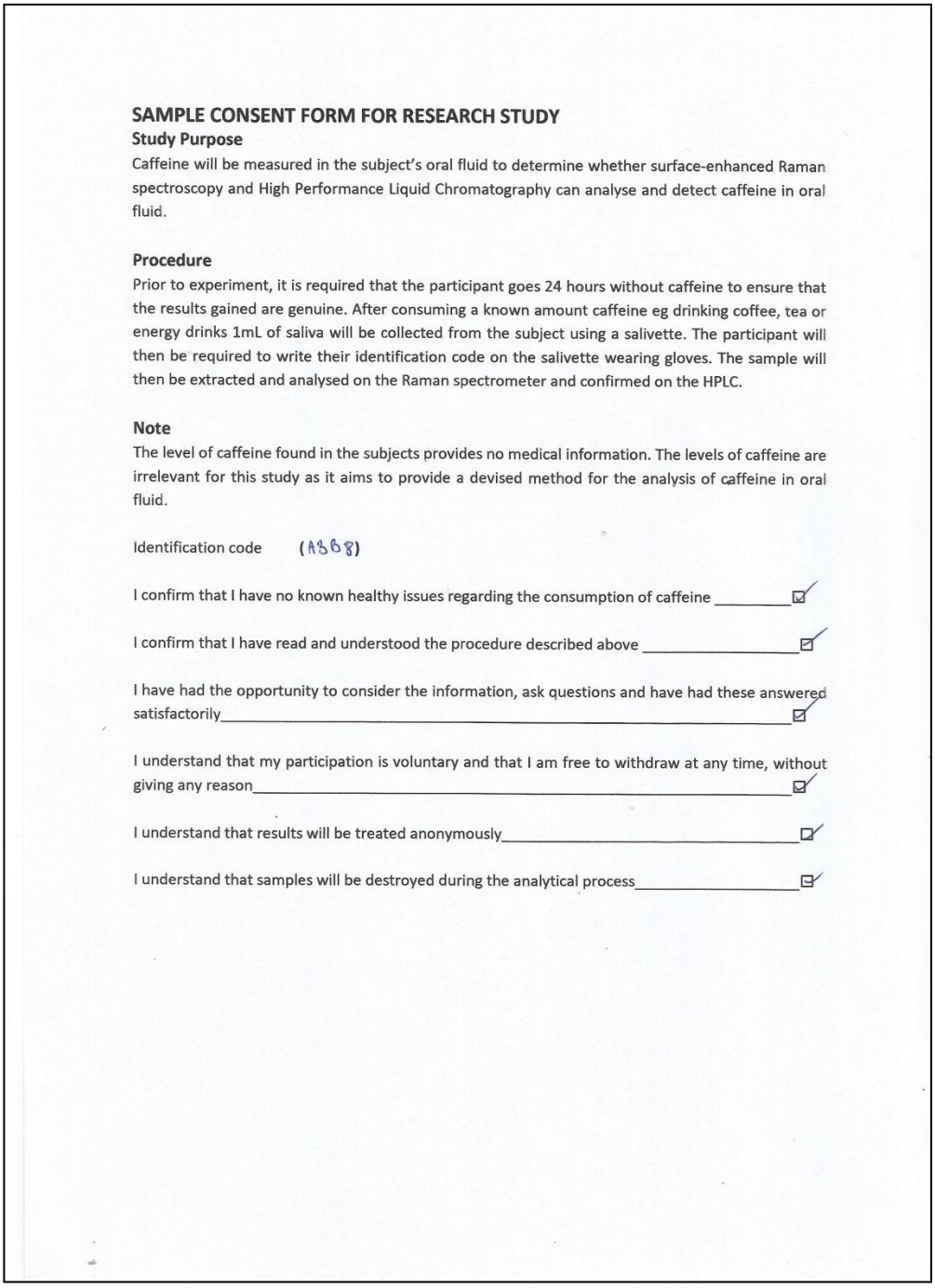
8. Appendices

Appendix 1: Example of the consent form each participant filled out



SAMPLE CONSENT FORM FOR RESEARCH STUDY

Study Purpose

Caffeine will be measured in the subject's oral fluid to determine whether surface-enhanced Raman spectroscopy and High Performance Liquid Chromatography can analyse and detect caffeine in oral fluid.

Procedure

Prior to experiment, it is required that the participant goes 24 hours without caffeine to ensure that the results gained are genuine. After consuming a known amount caffeine eg drinking coffee, tea or energy drinks 1mL of saliva will be collected from the subject using a salivette. The participant will then be required to write their identification code on the salivette wearing gloves. The sample will then be extracted and analysed on the Raman spectrometer and confirmed on the HPLC.

Note

The level of caffeine found in the subjects provides no medical information. The levels of caffeine are irrelevant for this study as it aims to provide a devised method for the analysis of caffeine in oral fluid.

Identification code (1368)

I confirm that I have no known healthy issues regarding the consumption of caffeine _____ ☑

I confirm that I have read and understood the procedure described above _____ ☑

I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily_____ ☑

I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason_____ ☑

I understand that results will be treated anonymously_____ ☑

I understand that samples will be destroyed during the analytical process_____ ☑

Appendix 2: Equation used to calculate the caffeine present in saliva

Equation of the line:

- $y = 15685x - 3533$

Rearranged to:

- $y = \frac{(x + 3533)}{15685}$

Example $\text{Conc} = \frac{(24033.16 + 3533)}{15685} = 1.76\ \mu g/mL$